SURVEILLANCE FOR CHRONIC WASTING DISEASE AND OTHER INFECTIOUS AGENTS IN MULE DEER (*Odocoileus hemionus*) AND WHITE-TAILED DEER (*Odocoileus virginianus*) IN SOUTHERN SASKATCHEWAN

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Keywords: mule deer, *Odocoileus hemionus,* white-tailed deer, *Odocoileus virginianus,* chronic wasting disease, survey, serology, parasites, rhadinovirus

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ABSTRACT

Chronic wasting disease (CWD) was detected in Saskatchewan wild deer populations in 2000 which prompted disease management actions consisting of population reduction. Little is known about population structure, health status, interactions or movement patterns of deer in Saskatchewan and these factors are important in designing a management program for CWD. As part of an ongoing study on deer movement patterns of wild deer in southern Saskatchewan, a survey was conducted to: 1) determine prevalence of CWD and selected infectious agents in mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*), and 2) identify infectious agents which could be used as a surrogate measure of the effectiveness of the adopted CWD management strategies. Tonsil biopsies, feces and blood were collected from 254 mule deer and 43 white-tailed deer during winters of 2006, 2007 and 2008. Immunohistochemical staining of tonsil biopsies for CWD revealed a prevalence of 2.4% (6/249) in mule deer and 0% (0/43) in white-tailed deer. Parasitological investigation of 253 fecal samples from mule deer identified eggs of nematodes in the superfamily Trichostrongyloidea (29.2%); and parasitic stages of the following genera: *Nematodirus* (7.1%), *Skrjabinema* (14.3%), *Trichuris* (0.8%), *Moniezia* (16.2%), *Thysanosoma* (12.2%), *Orthostrongylus* (35.2%), *Eimeria* (13.4%) and *Giardia* (0%, 0/137). A similar investigation of 42 white-tailed deer fecal samples identified parasitic stages of nematodes in the super family Trichostrongyloidea (4.8%) and in genera of *Orthostrongylus* (2.4%), *Moniezia* (2.4%) and *Eimeria* (2.4%). Dorsal-spined larvae were detected in 2.4% of the white-tailed deer fecal samples. In serum samples from 253 mule deer, antibodies (Ab) were detected against bovine herpesvirus1 (BoHV-1) (34.8%), parainfluenza-3 (PI-3) (56.5%), bovine virus diarrhoea virus (BVDV-1) (30.8%) and *Neospora caninum* (15.4%, 36/245). In serum samples from 40 white-tailed deer, Ab to BoHV-1(32.5%), PI-3 (35%), BVD-1 (12.5%) and *Neospora caninum* (20.5%, 8/39) was detected.

Based on relative host specificity, moderate prevalence and horizontal routes of transmission, herpesvirus, parainfluenza 3, *Eimeria* and *Skrjabinema* were identified as infectious agents which could potentially be used to evaluate the effectiveness of disease management strategies, which may in turn predict the response of CWD to these same strategies. Using polymerase chain reaction (PCR) a herpesvirus was detected, in 42.1% (40/95) of retropharyngeal lymph nodes from hunter-submitted mule deer and white-tailed deer heads from Saskatchewan in 2007. DNA sequences of the partial DNA polymerase gene from this virus were 98 - 100% identical to mule deer lymphotropic herpesvirus (mule deer-LHV). A 3.6 kb contiguous sequence of mule deer-LHV genome was generated by genome walking (GenBank Accession number: HM014314). Use of a mule deer-LHV-specific PCR on buffy coat samples collected during winters of 2007 and 2008, detected mule deer-LHV in 42.1% (67/158) of mule deer and 33.3% (8/24) of white-tailed deer. Very little DNA sequence diversity in the partial sequences of glycoprotein B (gB) gene and the intergenic spacer regions between DPOL and gB gene of mule deer-LHV was observed among deer from different wildlife management zones.

Mule deer-LHV is also a potential marker for evaluating the effectiveness of disease management activities because of its moderate prevalence, host specificity, ease of sample collection and the availability of a rapid and low-cost method for its detection. A variable region of the mule deer-LHV genome needs to be identified if this virus to be used as an inferential tool for studying host population structure.

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DEDICATION

To my mother and father, who cherished my life with their blessings

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LIST OF ABBREVATIONS

Ab	antibodies
BoHV-1	bovine herpesvirus 1
bp	base pairs
BVDV-1	bovine virus diarrhoea virus 1
CCWHC	Canadian Cooperative Wildlife Health Center
CI	Confidence Interval
CJD	Creutzfeldt-Jakob Disease
CNS	central nervous system
CWD	chronic wasting disease
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPOL	DNA polymerase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
epg	eggs per gram
FAT	fluorescent antibody technique
FBS	fetal bovine serum
g	gravitational force units (relative centrifuge force)
gB	glycoprotein B
h.	hour(s)
HRZ	herd reduction zone
IC	intracerebral inoculation

IFAT	indirect fluorescent antibody test
IHC	immunohistochemistry
ISR	intergenic spacer region
ITS	internal transcribed spacer
IU	international unit
kb	kilobases
km	kilometer
LHV	lymphotropic herpesvirus
lpg	larvae per gram
MCF	malignant catarrhal fever
min.	minute(s)
ml	milliliter
mm	millimeter
NCBI	National Center for Biotechnology Information
nm	nanometers
mM	millimoles
OD	optical density
opg	oocytes per gram
OR	Odds Ratio
PCR	polymerase chain reaction
pH	minus the decimal logarithm of hydrogen activity in aqueous solution
PI	percentage inhibition
PI-3	parainfluenza 3

PMCA	protein misfolding cyclic amplification
PRNP	prion protein
PrP^{C}	cellular prion protein
PrP^{CWD}	chronic wasting disease infectious prion protein
PrP^{res}	protease resistant prion protein
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RuRV	ruminant rhadinoviruses
sec.	seconds
SNT	serum neutralization test
TSEs	transmissible spongiform encephalopathies
U	units
v/v	volume per volume
WB	western blot
WMZ	wildlife management zone
w/v	weight per volume
µg	microgram
µl	microliter
µm	micrometer
µM	micromole
pmol	picomole
$^{\circ}C$	degrees centigrade

INTRODUCTION

Chronic wasting disease (CWD) is a naturally-occurring, fatal, neurodegenerative disease of North American cervids (Salman, 2003). CWD is a transmissible spongiform encephalopathy (TSE) caused by an infectious prion protein (PrP^{CWD}) and, like scrapie, is contagious. It is the only TSE known to occur in free-ranging species. CWD has been reported in game farms (Kahn et al., 2004), research facilities (Williams and Young, 1980), zoological gardens (Williams and Young, 1992; Dubé et al., 2006), and wildlife (Williams and Miller, 2002). CWD is transmitted among cervids through direct contact and persistent environmental contamination (Miller et al., 2000). CWD is important because of: 1) effects on free-ranging native cervids of North America; 2) cost of disease control measures in wild and captive North American cervids; 3) effects of disease and control measures on cervid hunting and wildlife recreational activities; and (4) potential implications for livestock and human health (Bourne, 2004).

CWD was introduced to Saskatchewan, Canada, in late 1980s and early 1990s through importation of infected farmed elk (*Cervus elaphus*) from USA (Kahn et al., 2004). The first diagnosed case of CWD in Saskatchewan was in 1996 in a farmed elk (Kahn et al., 2004). CWD was first detected in Saskatchewan free-ranging mule deer (*Odocoileus hemionus*) in 2000, and was subsequently detected in wild white-tailed deer (*Odocoileus virginanus*) and elk (Bollinger et al., 2008). Presently, CWD is known to be enzootic in certain geographical areas of Saskatchewan, specifically: 1) Manitou Sand Hills located south of Lloydminster, 2) Bronson Forest north and east of Lloydminster, 3) the South Saskatchewan River valley from north and

east of Swift Current to eastern Alberta, and 4) the boreal forest fringe near Nipawin, Saskatchewan (Bollinger et al., 2008).

In Saskatchewan, CWD in wild cervid populations has been managed by reducing deer numbers in known infected areas through increased hunter harvest and surveillance (Bollinger et al., 2008). Surveillance is intended to detect new foci of infection, estimate prevalence and evaluate effectiveness of adopted management strategies (Williams et al., 2002a). The effects of the current management strategies will not be evident for several years because of the long incubation period of CWD and extreme resistant nature of prions in the environment (Miller et al., 2000). Hence, alternative approaches for more rapid evaluation of current management strategies are needed. Other infectious agents of deer that have similar transmission modes to that of CWD, while having shorter incubation periods and therefore a shorter response time, are a potential tool in the evaluation of present management strategies.

Disease dynamics and population structure are important parameters in understanding the geographic spread of disease agents within a host population (Wasserberg et al., 2009). Population genetics can help elucidate this structure but is limited by the relative stability of vertebrate host genes and the frequently slow rates of evolutionary change; thus, host genetics may not reflect important population structure characteristics in recently introduced or expanding populations (Biek et al., 2006). Studying genetic variability of microparasites, which have shorter generation times and higher mutation rates than their hosts, is a more appropriate tool to understand host population structure in "shallow" time (Biek et al., 2006).

A survey of infectious agents in wild deer populations in southern Saskatchewan was undertaken in conjunction with a study on deer movement. The objectives of this survey were to:

1. Determine the prevalence of CWD and other selected infectious agents in deer populations in southern Saskatchewan
2. Identify infectious agents whose prevalence are likely to respond rapidly to herd reduction and other disease management actions, and therefore could be used as a tool in evaluation of CWD management strategies.
3. Determine a suitable candidate from selected pathogens that could be used to study pathogen spread, deer movement and population structure.

The first chapter reviews the available information on CWD with an emphasis on the interface between molecular epidemiology and population genetics. This chapter also reviews the use of viruses as inferential tools in studying host population structure. The second chapter describes the prevalence of CWD, endoparasites and selected viruses in wild deer populations of southern Saskatchewan. Infectious agents that could be potentially used in evaluating current disease management strategies are also discussed in this chapter. Chapter 3 describes prevalence and detailed molecular biological aspects of mule deer lymphotropic herpesvirus (mule deer-LHV) in different wildlife management zones, and its potential to be used as a tool in understanding the spatial spread of a disease agent among deer populations in Saskatchewan. Chapter 4 describes the identified infectious agents as potential tools to evaluate the CWD management strategies, and mule deer-LHVas a potential tool in inferring deer population structure.

1.0. LITERATURE REVIEW

1.1. Chronic wasting disease

Transmissible spongiform encephalopathies (TSEs) are a diverse group of fatal, neurodegenerative diseases characterized by the accumulation of protease resistant prion protein (PrP^{res}), an abnormal disease-specific isoform of cellular prion protein (PrP^{C}), in the central nervous system (CNS) (O'Rourke et al., 2004). TSEs are reported in a wide range of animal species and include: scrapie of sheep (*Ovis aries*) and goats (*Capra hicus*); transmissible mink encephalopathy in farmed mink (*Mustela vison*); Creutzfeldt-Jakob disease (CJD), kuru, fatal familial insomnia, Gerstmann Straussler-Scheinker syndrome, and variant CJD in humans; bovine spongiform encephalopathy in cattle (*Bos taurus*); feline spongiform encephalopathy of domestic and exotic cats; and chronic wasting disease (CWD) in cervids (Gavier-Widen et al., 2005). Of the TSEs, only scrapie and CWD are contagious, and CWD is the only TSE to affect free-ranging species (Spraker et al., 1997; Williams and Miller, 2002). CWD has emerged as an important disease of wildlife in North America and of deer and elk kept in captivity (Williams and Miller, 2002; Williams et al., 2002a).

1.1.1. History of chronic wasting disease

Chronic wasting disease was first reported in 1967 at a research facility in Colorado, USA, as a clinical syndrome of unknown etiology in mule deer (*Odocoileus hemionus*) (Williams and Young, 1980). It was also reported in a research facility in Wyoming, USA, in 1978 (Williams and Young, 1992). In 1979, CWD was diagnosed in Rocky Mountain elk (*Cervus elaphus nelsoni*) in a Colorado research facility (Williams and Young, 1982). Since

then, the disease has been reported in free-ranging cervids in USA: elk in 1981 (Spraker et al., 1997), mule deer in 1985, white-tailed deer (*Odocoileus virginianus*) in 1990 (Williams and Miller, 2002), and moose (*Alces alces*) in 2007 (Baeten et al., 2007).

In Canada, CWD was first reported in 1996 in a farmed elk in Saskatchewan (Williams and Miller, 2002). CWD has been reported in farmed cervids in only two provinces, Alberta and Saskatchewan, with 95% of the affected farms located in Saskatchewan (Kahn et al., 2004). During 1996 to 2002, CWD was diagnosed in 39 captive elk herds in Saskatchewan (Argue et al., 2007). Epidemiological investigations of these cases revealed imported elk from South Dakota, USA, as the source of infection (Williams et al., 2002a). The first case of CWD in a farmed white-tailed deer was reported from Alberta in 2002 (Kahn et al., 2004). In Canada, CWD was classified as a reportable disease in 2002 (Dubé et al., 2006). To date, CWD has been reported in free-ranging cervids in Saskatchewan and Alberta, with the first cases in Saskatchewan and Alberta occurring in 2000 and 2005, respectively, and both cases reported in wild mule deer (Wilson et al., 2009). The initial case of CWD in wild elk was reported in 2008 (Bollinger et al., 2008). Surveillance of free-ranging cervids in Saskatchewan by the Saskatchewan Ministry of the Environment and the Canadian Cooperative Wildlife Health Centre (CCWHC) during 1997 – 2008 revealed that 7.7/1000 of mule deer (189/24457), 3.9/1000 of white-tailed deer (54/13975) and 2.3/1000 of elk (3/1318) were positive for CWD (Bollinger et al., 2008).

A retrospective study on cervid tissues from the Toronto Zoo, Ontario, revealed CWD in seven mule deer and a black-tailed deer (*Odocoileus hemionus columbianus*) that died in late 1970s and early 1980s. The origin of CWD in these cases was most likely the importation of deer from a US zoo (Dubé et al., 2006). The only reported case of CWD outside North America

was in South Korea, and this was an elk imported from Saskatchewan in 1997 (Sohn et al., 2002; Williams et al., 2002a).

1.1.2. Etiology of chronic wasting disease

Initially, cases of CWD were thought to be due to stress, nutritional deficiencies or intoxication (Salman, 2003). In 1978, CWD was identified as a form of TSE (Williams and Young, 1980), and a poorly characterized, infectious prion protein was identified as the causative agent (Prusiner, 1982; Williams and Miller, 2002). The hypothesized origins of CWD are as follows: 1) a spontaneous somatic mutation of the prion protein (PRNP) gene of mule deer, and subsequent spread to other cervid species; 2) a strain of scrapie that adapted to cervids; or 3) infection of cervids with an unrecognised prion strain (Williams and Miller, 2002, 2003). According to the prion hypothesis, infectious prion proteins are generated by conversion of normal prion protein to its misfolded form which is associated with a change in the tertiary structure with a decreased α-helical content and an increased β-pleated sheets (Prusiner, 1997). This post-translational modification of normal cellular prion protein to its abnormal, infective isoform is believed to be the molecular basis of prion diseases (Gavier-Widen et al., 2005). The generated, protease-resistant, prion proteins (PrP^{res}) are extremely resistant to environmental conditions, and most physical and chemical disinfectants (Taylor, 2000).

Identification of PrP^{res} of CWD (PrP^{CWD}) and other TSEs is based on conformation-dependent immunoassays, and bioassays performed in transgenic and wild mice (Safar et al., 2002). A bioassay on transgenic mice revealed that the tested CWD strains from deer and elk were similar in incubation periods, histopathological findings and biochemical properties of PrP^{sc} (Browning et al., 2004). The marked similarity of pathological changes in the CNS and their similar epidemiology and glycoform patterns strongly suggest that PrP^{CWD} in captive and

free-ranging deer and elk are identical (Williams and Miller, 2002); however, immunoblot quantification studies on retropharyngeal lymph nodes and tonsils revealed that these tissues from deer contained more PrP^{res} than did those from elk (Race et al., 2009).

1.1.3. Host range for chronic wasting disease

The natural host range of CWD appears to be limited to mule deer, white-tailed deer, Rocky Mountain elk and moose (Salman, 2003; Sigurdson and Miller, 2003). Since infectious agents of wild mule deer and white-tailed deer were investigated, an emphasis is given on their social structure and behavioural characteristics.

1.1.3.1. Mule deer

Mule deer (*Odocoilus hemionus*) belong to the order Artiodactyla, suborder Ruminantia and family Cervidae (Kie and Czech, 2000). Mule deer evolved before humans arrived in North America, and inhabit western Canada, Mexico and the USA (Kie and Czech, 2000). There are nine subspecies of mule deer, and Rocky mountain mule deer is the only subspecies found in Saskatchewan, Canada (Kie and Czech, 2000). Mule deer is an important big game species, and play an important role in ecosystem structure and function. Mule deer groups are lead by dominant females, and males disperse as individuals or as groups of unrelated individuals (Geist, 1981). They are polygynous breeders, females breed at the age of 1.5 years, and the number of foetuses per yearling female and adult female is 0.66 – 1.71 and 0.92 – 1.96, respectively (Kie and Czech, 2000). In montane environments they migrate to higher elevations in the summer and

descend to lower elevations with the snow fall (Nicholson et al., 1997). Their home range size varies with sex, age, body mass, season and habitat (Anderson and Wallmo, 1984).

1.1.3.2. White-tailed deer

White-tailed deer (*Odocoilus virginianus*) belong to the order Artiodactyla, suborder Ruminantia and family Cervidae (Demarais et al., 2000). White-tailed deer are the most widely distributed mammal in North America, and occur from Northwest territories and southern provinces of Canada in North America to Peru and Brazil in South America (Demarais et al., 2000). There are 38 known subspecies of white-tail deer, and subspecies *Odocoileus virginianus dactotensis* occur in Saskatchewan, Canada (Demarais et al., 2000). They are polygynous in breeding behaviour (Miller, 1970), and yearling females produce a single fawn while the adults produce twins or triplets (Demarais et al., 2000). Yearling males disperse from their natal areas to join bachelor groups (Miller, 1970). White-tailed deer have female dominant social units and and congregate in coniferous forests during winter to access thermal cover, reduced wind velocities and decreased snow depth (Demarais et al., 2000).

1.1.4. Experimental and field observations of chronic wasting disease in cervids

1.1.4.1. Incubation period of chronic wasting disease in cervids

The incubation period of naturally occurring CWD in free-ranging cervids is not known (Spraker et al., 2004). The earliest age in which clinical signs of CWD in deer has been observed is 18 months, and has been suggested to be the minimum incubation period (Williams and Young, 1992). In experiments with PrP^{res} introduced orally to mule deer, the minimum incubation period was 15 months, with a mean period from exposure to death of 20 - 25 months

(Williams et al., 2002a). In a Saskatchewan game farm, CWD was confirmed in an elk less than 12 months of age by immunohistochemistry (IHC) (Kahn et al., 2004). Further, a variable incubation period of 12 - 34 months has been observed in Rocky Mountain elk following oral administration of PrP^{res} (Williams and Miller, 2002).

1.1.4.2. Pathogenesis of chronic wasting disease

Pathogenicity studies on CWD have been conducted in mule deer and elk by oral administration of brain homogenates from conspecific clinical cases (Sigurdson et al., 1999; Williams and Miller, 2002; Spraker et al., 2004). Experimental studies on CWD in mule deer have revealed a consistent pattern of PrP^{res} deposition initially in tonsil and gut-associated lymphoid tissue, then in the enteric nervous system, followed by the CNS at the vagal nucleus and thoracic spinal cord with concurrent distribution to peripheral lymphoid tissues (Sigurdson et al., 1999; Miller and Williams, 2002; Spraker et al., 2002b). Experimental infection of mule deer fawns revealed the presence of PrP^{res} in retropharyngeal and ileocecal lymph nodes, Peyer's patch and tonsil at 42 days post inoculation (Sigurdson et al., 1999). Once infected, deer appear to shed PrP^{res} persistently until death (Gross and Miller, 2001).

In natural cases of CWD, the parasympathetic vagal nucleus of the medulla oblongata has been reported to be the site of consistent lesions (Williams and Young, 1993). PrP^{res} has also been detected in brain, palatine tonsil, visceral and peripheral lymph nodes, and lymphoid tissue of the small and large intestine (Sigurdson et al., 1999; Salman, 2003).

1.1.4.3. Clinical signs of chronic wasting disease

Most CWD cases detected in surveillance programs are sub-clinical, and detection of clinical disease in free-ranging cervids is relatively rare (Williams, 2005). Clinical signs of CWD appear at the terminal stages of the disease (Williams et al., 2002a). In deer and elk, the most prominent clinical features are loss of body condition and behavioural changes (Williams and Miller, 2002). With progression of clinical disease, animals develop polydipsia, polyuria, ataxia, dehydration, salivation, drooping head and ears, loss of coordination, and fine head tremors (Williams and Young, 1980; Williams and Miller, 2002). The clinical course of CWD varies from a few days to approximately one year, and the duration is likely to be shorter in free-ranging deer and elk compared to those in captivity (Williams and Miller, 2002). Once deer are infected, the disease is progressive and invariably fatal (Gross and Miller, 2001).

1.1.4.4. Pathology

Gross lesions of CWD are non-specific; affected animals may have a rough, dry hair coat which fails to shed (Williams, 2005). A healthy body condition may not exclude affected animals, but typically, in terminal stages of the disease, affected animals are emaciated, have megaeosophagus and their ruminal contents are watery with increased amounts of gravel and sand. Urine may be dilute if there is access to water and if not, the carcass may be dehydrated. Aspiration pneumonia may be detected at necropsy (Williams and Miller, 2002).

Microscopic lesions of CWD in the CNS are characteristic of the TSEs and are similar in distribution in deer and elk (Williams and Miller, 2002; Williams, 2005). Accumulation of PrP^{res} in nervous tissue is associated with microcavitation of grey matter; degeneration and

intracytoplasmic vacuolation of neurons; and spongiform changes in neurons of the spinal cord, medulla oblongata, pons, mesencephalon, thalamus, hypothalamus and cerebellar cortex (Williams and Young, 1982). Amyloid plaques may be detected in haematoxylin and eosin stained brain sections (Williams, 2005), but their detection is improved with Congo Red (Bahmanyar et al., 1985). Hyperplasia and hypertrophy of astrocytes have also been observed in CNS (Gavier-Widen et al., 2005).

Lymphoid tissue is also useful in diagnosis of CWD as PrP^{res} accumulates in this tissue (Gavier-Widen et al., 2005); however, a differences between deer and elk in quantities of PrP^{res} in tonsils and lymph nodes has been reported (Race et al., 2007). It is important to test lymphoid follicles from the cortex of lymph nodes as this is the site of prion accumulation (Gavier-Widen et al., 2005). Antibody response against PrP^{res} has not been reported (Williams et al., 2002a).

1.1.5. Susceptibility of other animals to chronic wasting disease

A broad range of animal species developed CWD intracerebrally (IC) with brain homogenate from CWD-infected deer (Williams and Miller, 2002; Sigurdson and Miller, 2003). These species include: domestic ferret (*Mustela putorious*) (Bartz et al., 1998; Sigurdson et al., 2008), American mink (*Mustela vison*), squirrel monkey (*Saimiri sciurius*) (Marsh et al., 2005), domestic goat (Williams and Young, 1992), cattle (Hamir et al., 2001; Hamir et al., 2005), domestic sheep (Hamir et al., 2006); meadow voles (*Microtus pennsylvanicus*), red-backed voles (*Myodes gapperi*), white-footed mice (*Peromyscus leucopus*) and deer mice (*Peromyscus maniculatus*) (Heisey et al., 2010), and laboratory rodents (Williams and Young, 1992). Inter-species transmission of CWD is supported by experimental reproduction of CWD by IC inoculation of brain homogenate of CWD-infected mule deer passaged in ferret to hamsters

(*Mesocricetus auratus*) (Bartz et al., 1998). In contrast, IC inoculation of CWD-infected mule deer brain homogenate to raccoons (*Procyon lotor*) (Hamir et al., 2003), and IC and oral inoculation of CWD infected brain homogenates to Cynomolgus macaques (*Macaca fascicularis*) failed to produce disease after five years (Race et al., 2009).

1.1.6. Animal models and *in vitro* studies of chronic wasting disease

Transmission of CWD to cervid PrP-expressing transgenic mice has been successful, and these infected mice have developed plaques in the brain similar to those present in CWD affected cervids (Browning et al., 2004). This transgenic mouse model is used to study various aspects of CWD (Kong et al., 2005; Trifilo et al., 2007; Race et al., 2009). A transformed cell line originating from a CWD infected mule deer brain tissue has been used to study the inhibitors of CWD infection (Raymond et al., 2006) and CWD infectivity (Sigurdson and Aguzzi, 2007). This *in vitro* method may replace the *in vivo* bioassay for prion infectivity and end-point titrations due to the high susceptibility of the transformed cell line to prion proteins (Bosque and Prusiner, 2000).

1.1.7. Public health importance

Human susceptibility to CWD is not clear, but the risk appears to be low (Salman, 2003; Sigurdson and Aguzzi, 2007). Susceptibility of humans to CWD has been studied experimentally in transgenic mice expressing the human prion protein (PRNP) gene; CWD infection in these mice was not evident up to 756 days post-inoculation (Kong et al., 2005). An *in vitro* study evaluating the existence of a molecular barrier limiting susceptibility of humans, cattle, cervids

and sheep to various TSEs demonstrated, that although humans are less susceptible to CWD than the natural hosts, they are not completely resistant to *in vitro* conversion of PrP to PrP^{res} (Raymond et al., 2000).

Hunters in USA and Canada have been exposed to CWD by consumption of venison; but, to date, there has been no observed correlation between venison consumption and CJD (Sigurdson and Aguzzi, 2007). Due to the uncertainty of the human health risk, the recommendation of World Health Organization and North American health authorities is to avoid consumption of TSE infected meat (Salman, 2003). In Canada, all adult cervids slaughtered in commercial facilities of Saskatchewan, Manitoba and Alberta are tested for CWD, and only CWD-negative meat is released for consumption (Salman, 2003).

1.1.8. Transmission and epidemiology of chronic wasting disease

1.1.8.1. Transmission of chronic wasting disease

Among the TSEs CWD is transmitted most efficiently in susceptible species (Sigurdson and Aguzzi, 2007), and is thought to be both contagious and infectious; however, the exact mode of transmission has not yet to be elucidated (Salman, 2003). The disease is reported to be transmitted horizontally via host-to-host contact or environmental contamination (Spraker et al., 1997); and vertically from doe to fawn (Miller et al., 2000). Horizontal transmission by prion-infected secretions and excretions such as saliva, feces and urine (Miller et al., 1998; Sigurdson et al., 1999; Haley et al., 2009), as well as by decomposed carcasses is thought to be important in the spread of CWD (Salman, 2003; Sigurdson and Aguzzi, 2007). In mule deer fawns, detection of PrP^{res} in retropharyngeal lymph nodes, Peyer's patch, tonsil and ileocaecal lymph nodes forty-

two days following oral inoculation suggests the possibility of excretion in saliva and feces before the onset of clinical disease (Sigurdson et al., 1999). Investigations of infected elk farms in Saskatchewan support horizontal transmission as the primary route in CWD epidemiology (Kahn et al., 2004). Maternal transmission, if it occurs, may not play a significant role in the epidemiology of CWD (Miller et al., 2000). Histological and IHC examinations of tissues from a fetus and placentomes of a CWD-infected doe were reported to be negative for PrP^{res} and spongiform lesions (Spraker et al., 2002c).

Interspecies transmission of CWD among mule deer, elk, and white-tailed deer is also suspected (Williams and Miller, 2002). Due to the presence of greater amounts of PrP^{res} in tonsil and retropharyngeal lymph nodes of deer than those of elk, deer are more likely to transmit CWD to other cervids (Race et al., 2007). Transmission of CWD from an infected to a susceptible cervid is thought to require a longer exposure time than a brief, transient interaction (Williams et al., 2002a; Bollinger et al., 2004).

Transmission of CWD occurs more readily in animals kept in captivity than those in free-ranging populations (Williams et al., 2002a; Race et al., 2007). Sharing common salt licks and water in captivity may enhance CWD transmission (Sigurdson and Aguzzi, 2007). Being social animals, deer tend to stay in herds, yard together in winter, and engage in physical contact; these behaviour patterns may contribute to a high risk of exposure to CWD within infected populations in the wild (Lupi, 2005). Differences in social interactions, home range size, and preferred habitat might explain detected differences in prevalence of CWD among cervid species in natural settings (Race et al., 2007). Supplemental feeding of free-ranging deer concentrates animals at feeding sites, facilitates close contact, and thereby enhances spread of infectious diseases through saliva, feces or urine (Thompson et al., 2008).

Accumulation of PrP^{res} in the gut-associated lymphatic tissues (tonsil, Peyer's patch and mesenteric lymph nodes) of an infected host suggests that excretion may occur through the alimentary tract (Miller and Williams, 2003), favouring environmental contamination. Contaminated pasture may act as a source of infection in the spread of CWD (Williams and Miller, 2002). Soil becomes contaminated with PrP^{res} by excreta of infected animals (Lupi, 2005) and by decomposition of infected carcases (Miller et al., 2004; Sigurdson and Aguzzi, 2007). PrP^{res} is also known to adsorb to soil particles and interact avidly with aluminosilicate clay mineral (Johnson et al., 2006); prion bound soils retains infectivity for years (Johnson et al., 2006; Genovesi et al., 2007). Cervids ingest soil accidentally during grazing or intentionally to satisfy their salt requirements (Lupi, 2005), a habit which may increase their risk of exposure to CWD. Accumulation of PrP^{res} in soil creates an environmental reservoir for indirect transmission (Miller et al., 2004). Detection by protein misfolding cyclic amplification (PMCA) of low levels of PrP^{CWD} in water samples from a CWD-endemic area in the USA supports the idea of persistent accumulation of prions in the environment (Nichols et al., 2009). Environmental contamination is a potential reservoir of PrP^{res} for maintaining epidemics of CWD (Miller and Williams, 2003). Existence of PrP^{res} for a decade following depopulation and decontamination of a CWD-infected deer research facility in Colorado, USA demonstrates its resistance and ability to remain in the environment in the absence of its host (Williams and Miller, 2003). CWD has been reproduced experimentally by exposure of CWD-negative mule deer to CWD-infected deer, environments contaminated with CWD, and decomposed carcasses of CWD-infected deer (Miller et al., 2004). The possibility of additional vertebrate or invertebrate species acting as reservoirs of CWD has been suggested, but will require further study (Salman, 2003; Lupi, 2005).

1.1.8.2. Epidemiology of chronic wasting disease

The epidemiology of CWD in free-ranging cervids is poorly understood (Gross and Miller, 2001). The origin of CWD in the initially detected research facility in Colorado, USA, is unclear because of mixing of captive and wild deer populations within the facility (Williams and Miller, 2003). However, in Saskatchewan, Canada, spill-over from infected game farms to wildlife is thought to be the origin of CWD in free-ranging cervids (Bollinger et al., 2004). CWD is reported in several focal areas separated by large distances (Sigurdson and Aguzzi, 2007). Natural movement of free-ranging deer in the wild, transportation of infected captive cervids, and other unknown risk factors may have likely contributed to the geographic expansion of CWD (Miller and Williams, 2004). The prevalence of CWD is lower in recently introduced areas than in long standing endemic areas (Miller and Conner, 2005).

Variability in incubation period and age at infection may explain the wide range of ages of cervids infected with CWD (Spraker et al., 1997); however in experimental transmission studies in deer, susceptibility to CWD is similar between the sexes and across age classes (Williams and Young, 1980; Williams and Miller, 2002). Increased interactions during the breeding season, and grouping of males prior to the rut may contribute to the higher prevalence observed in free-ranging male mule deer (Miller and Conner, 2005). These interactions are likely to drive overall patterns of large-scale geographic spread (Conner and Miller, 2004). However transmission of CWD between closely related female deer could be an important in the spread of CWD locally (Grear et al., 2010). Temporal, spatial and demographic factors contribute to differences in CWD prevalence in free ranging mule deer in Colorado (Miller and Conner, 2005). Although CWD is reported throughout the year, there is an observational bias as it is most

commonly diagnosed during surveillance of hunter harvested animals in the fall (Williams, 2005).

1.1.9. Diagnosis of chronic wasting disease

Diagnosis of CWD based upon clinical signs is inappropriate due to their variability and lack of specificity (Miller and Williams, 2004). Diagnosis of CWD is challenging due to the long incubation period and uncertainty regarding the time period following infection to the appearance of PrP^{res} in tissues (Wild et al., 2002). Several tests are available for the diagnosis of CWD; however the choice of the diagnostic test depends on the purpose of testing, i.e. screening or confirmation of CWD (Gavier-Widen et al., 2005).

Diagnosis of clinical CWD is made by detection of spongiform lesions in the brain and spinal cord by histopathology (Williams and Young, 1980), and confirmed by detection of widespread PrP^{res} in CNS tissues by immunological techniques. Detection of CWD infection depends on the demonstration of PrP^{res} in follicles of tonsils, lymph nodes and/or medulla oblongata (Spraker et al., 2002c; Wild et al., 2002; Williams et al., 2002a; Gavier-Widen et al., 2005). Difficulties in detecting lesions in tissues after autolysis (Kahn et al., 2004) and delays in development of histological lesions (Williams and Miller, 2002) limit the usefulness of histopathology in the diagnosis of preclinical CWD.

Immunohistochemistry (IHC) is considered as the "gold standard" for the diagnosis of CWD (O'Rourke et al., 2003; Salman, 2003). It is advantageous over histopathology due to the ability to diagnose CWD in autolyzed samples (Spraker et al., 2002a), and in tissues with no detectable histopathological lesions (Sigurdson et al., 1999). Dorsal motor nucleus of the vagus nerve at the level of the obex in medulla oblongata is the consistent neuro-anatomical site for detection of CWD in deer and elk (Peters et al., 2000; Spraker et al., 2002b). Tonsils or

retropharyngeal lymph nodes of deer also may be tested with IHC in order to diagnose CWD and have the advantage of detecting infection early in the incubation period (Spraker et al., 2002b); these are the tissues commonly used in surveillance of hunter-harvested deer. As monoclonal Ab are incapable of discriminating between cellular prion protein (PrP^{C}) and PrP^{res}, specificity of this method depends on pre-treatment of tissues (Williams and Miller, 2002).

Testing of tonsillar biopsies is a valuable method for preclinical detection of CWD in deer (O'Rourke et al., 2003), which potentially could be used as a management tool for controlling CWD (Salman, 2003). However, this method requires restraint and anaesthesia (Williams, 2005), which limits its application to large surveillance programs and research (Wolfe et al., 2002). Examination of a minimum of four to six follicles (Gavier-Widen et al., 2005) or more than nine follicles (Wolfe et al., 2002), is required to provide a reliable negative result for a tonsil biopsy because the initial distribution of PrP^{res} in this tissue is not homogenous. This method is not useful in elk, as accumulation of PrP^{res} in lymph nodes occurs to a lesser degree in a greater proportion of animals than in deer (Wild et al., 2002). Rectal biopsy also has been found to be an effective preclinical CWD detection method in elk (Spraker et al., 2009) and white-tailed deer (Keane et al., 2009), and it does not require anaesthesia.

Other methods available for detection of CWD are enzyme-linked immunosorbent assay (ELISA) (Hibler et al., 2003), western blot (WB) (Guiroy et al., 1993), dot-blot assay (O'Rourke et al., 2003) and conformation-dependent immunoassay (Safar et al., 2002; Brooks et al., 2009). These tests are being used for screening, pathogenicity studies, and comparative studies at the molecular level (Gavier-Widen et al., 2005). Commercial ELISA kits for diagnosis of CWD have shown a high sensitivity and specificity, and allow rapid testing of large numbers of samples (Hibler et al., 2003; Gavier-Widen et al., 2005), which facilitates their use in

surveillance programs (Salman, 2003). ELISA and IHC are more sensitive than WB for detection of PrP^{res}, therefore, more suitable in diagnosis and surveillance (Race et al., 2007); however, WB is found to be more suitable for quantification or visualization of the banding pattern of PrP^{res} (Race et al., 2007). False-positives detected in rapid tests can be eliminated by IHC (Bollinger et al., 2004; Gavier-Widen et al., 2005). Protein misfolding cyclic amplification (PMCA) is a novel method which allows *in vitro* conversion of PrP^{c} to PrP^{res} in the presence of low levels of PrP^{res}, and could be used to detect relatively low quantities of PrP^{res} present in tissues and biological fluids (Saborio et al., 2001). PMCA has a very high sensitivity and specificity, and could be used in early diagnosis of prion diseases (Saá et al., 2006). This novel method has been applied in the diagnosis of CWD (Kurt et al., 2007).

1.2. Management and control of chronic wasting disease

Currently, there is no treatment for CWD-infected animals, or a vaccine to prevent infection (Williams et al., 2002a; Williams et al., 2002b; Kahn et al., 2004; Wasserberg et al., 2009), although several research studies with the goal of developing a vaccine have been conducted (Pilon et al., 2007; Goni et al., 2008). With the identification of CWD in an area, most jurisdictions have attempted to manage the disease through reduction of deer populations in order to decrease contact rates (Gross and Miller, 2001). These disease management programs should be designed within an experimental framework in order to improve our understanding of the epidemiology of CWD (Wasserberg et al., 2009). Containment and management of CWD is important because of its potential adverse effects on cervid populations (Gross and Miller, 2001), uncertain implications for human and domestic animal health and the unclear economic consequences of widespread occurrence of the disease (Wasserberg et al., 2009).

1.2.1. Management and control of chronic wasting disease in captive cervids

In USA, surveillance for CWD in farmed elk commenced in 1996 (Salman, 2003). Eradication is the goal of CWD management programs for captive deer and elk herds (Williams, 2005). Quarantine and depopulation of affected herds are options in managing CWD in captivity (Williams et al., 2002a) and game farms should be fenced to prevent contact between captive cervids and free-ranging animals (Williams and Miller, 2002). Importation of cervid species has been banned in several states of USA (Salman, 2003).

In Canada, quarantine and depopulation of infected herds, screening of all depopulated animals over 12 months of age, and tracing of all animals in contact with an infected herd are the methods employed to manage CWD on farms (Kahn et al., 2004). Animals which left the herd within 36 months of CWD being detected are traced, humanely killed, and tested; and animals which had left the herd between 36 and 60 months of the infection being detected on the farm are traced and monitored. Restocking of infected farms for variable time periods is also prohibited in order to prevent re-infection due to environmental contamination (Kahn et al., 2004).

1.2.2. Management and control of chronic wasting disease in wildlife

Managing CWD in the wild is far more challenging than in captivity (Williams and Miller, 2002). Eradication was the management strategy for CWD in free-ranging cervids when cases were reported initially, but with expansion of geographic distribution and host range, the strategy has changed to containment and prevalence reduction in endemic areas (Williams et al., 2002a). CWD is a relatively novel disease in free-ranging cervids, and management strategies are still in preliminary stages. These strategies should be focused on monitoring deer densities,

altering age structures of populations, decreasing the size of affected areas, and reducing transmission rates and disease prevalence (Bollinger et al., 2004).

Disease surveillance programs generate information on prevalence, spatial distribution and progression of disease (Nusser et al., 2008). Surveillance for CWD is widely practiced to determine prevalence in endemic areas, detect new foci of infection, and evaluate management strategies (Williams et al., 2002a). This is achieved by testing of tissues from deer killed by hunters and deer depopulated in targeted areas (Salman, 2003). Harvest-based surveillance programs are less effective than targeted surveillance in early detection of CWD (Miller et al., 2000). Accuracy of harvest location is also a limiting factor in most harvest-based surveillance systems (Bollinger et al., 2004). A targeted surveillance study suggested that prevalence of CWD in an area may be 1% or higher before clinical cases are initially detected (Miller et al., 2000). Surveillance programs should focus on testing of adults (> 1 year) to improve chances of detection (Bollinger et al., 2004). Long-term surveillance programs generate data on disease dynamics of CWD, and such data can be used to reinforce knowledge on CWD transmission and to design new management strategies (Miller et al., 2000; Gross and Miller, 2001).

Various strategies have been deployed to manage CWD in North America including ban on supplemental feeding, restrictions on translocation of cervids from endemic areas, selective culling of suspected clinical cases, and local population reductions (Williams et al., 2002a). Early aggressive intervention through selective culling or generalized population reduction has shown promise in preventing the establishment of new endemic foci (Gross and Miller, 2001); however, for existing endemic foci, the goal is to reduce the prevalence of CWD, as well as the amount of environmental contamination in the area (Bollinger et al., 2004). Selective and non-selective culling are important in eliminating infected deer, altering host density, and affecting

rates of disease spread and prevalence (Wasserberg et al., 2009). Selective culling may be more effective than random culling when a large proportion of the population can be tested, and early removal of preclinical infected individuals is possible (Wolfe et al., 2004); however, selective culling of clinically affected animals permits preclinical cases to remain in the population and contribute to environmental contamination (Williams and Miller, 2002). "Test-and cull" may be the most feasible method for controlling CWD in national parks and residential areas where urban and suburban deer are not harvested (Wolfe et al., 2002).

1.2.3. Constraints associated with management of chronic wasting disease

Subtle early clinical signs, long incubation periods, extreme resistance of the agent, environmental contamination, limited ability to detect preclinical cases, and an incomplete understanding of transmission dynamics, are all factors that hamper the management of CWD (Williams and Miller, 2002; Williams et al., 2002a; Kahn et al., 2004). These issues are further complicated by the extensive geographic range of deer and elk in the Americas, and the potential for CWD to infect new host species (Williams et al., 2002a). The cost of passive surveillance programs has also been identified as a management constraint (Williams et al., 2002a). When CWD is at low prevalence, a large sample size is required for its detection. Large sample size requirements may exceed sustainable harvest levels of the deer population, an issue that may negatively affect public support (Bollinger et al., 2004). Epidemiological models suggest that a long-term strategy is necessary for any program to be effective in managing CWD in free-ranging deer populations, which in turn requires a long-term commitment of personnel and funding (Gross and Miller, 2001; Wolfe et al., 2004).

1.3. Interface between epidemiology and population genetics

Epidemiology deals with disease dynamics in a population, while population genetics focuses on the inheritance of genes at the population level (Paterson and Viney, 2000). Within a population, inheritance of genes and transfer of infectious agents are analogous in many ways (Paterson and Viney, 2000). Association between ecology, spatial spread and evolutionary change in infectious diseases is of critical importance for understanding the extent of epidemiological patterns (Grenfell et al., 2004).

Advances in molecular techniques facilitate the analysis of population variation based on comparison of DNA sequence data at the nucleotide level (Pluzhnikov and Donnelly, 1996). Through natural selection, pathogens evolve in response to local host interactions and local environmental conditions to produce genetically distinct, locally-adapted ecotypes (Real et al., 2005). Genetic diversity is one of the most important characteristics of living organisms, and one which favours evolutionary changes (Rojas et al., 1993). Due to their high mutation rates and short generation times, rapidly evolving organisms are suitable candidates for studying genetic diversity (Duffy et al., 2008). Mutations generate pathogenic strains and phylogenetic lineages while epidemiological and immunological forces determine their relative survival (Grenfell et al., 2004).

Since macro- and microparasites have faster mutation rates and shorter generation times than their hosts, they often undergo significant population differentiation faster than that observed in the genetic system of any vertebrate hosts (Biek et al., 2006; Kitchen et al., 2008). When a homologous locus is compared between host and parasite, the rate of evolution of parasite DNA is faster than that of their host (Hafner et al., 1994). Hence parasites were also reported to be used as tools in population genetics and phylogeny of their hosts (Le et al., 2000;

Beveridge et al., 2007). Viruses with RNA genomes have mutation rates that are greater than those of viruses with DNA genomes (Simmonds, 2001); however, large DNA viruses still have faster rates of evolution than their hosts (Shackelton and Holmes, 2004). Geographical analysis of DNA sequences of these variants will aid in understanding patterns of disease emergence (Grenfell et al., 2004).

1.3.1. Viruses as inferential tools of host population structure

Viruses are obligatory intracellular parasites and their replication is ensured by modulation of biosynthetic machinery of the host cell (Bamford et al., 2005). The viral genome consists of double-stranded deoxyribonucleic acid (DNA), single-stranded DNA, positive-strand single-stranded ribonucleic acid (RNA), or negative-strand RNA and double stranded RNA (Hughes and Hughes, 2007). The viral envelope consists of a lipid bi-layer derived from a host cell, and surface glycoproteins encoded by the virus (Wiley and Skehel, 1987). The close association of a virus with its host exposes the virus to selection pressure exerted by the host's immune system (Hughes and Hughes, 2007). In this interaction, envelope glycoproteins are frequent targets for host Ab (Suzuki, 2006; Achour et al., 2008).

Viral genes can be classified as core, genus-specific, and species-specific genes (Shackelton and Holmes, 2004). Core genes are conserved among all family members, and are present in that family's common ancestor; genus-specific and species-specific genes are obtained subsequent to divergence of the genera or species (Shackelton and Holmes, 2004). Core genes of large-sized DNA viruses with linear genomes are located in the center of the genome, whereas genus- and species-specific genes are located close to the ends (Shackelton and Holmes, 2004). Core genes are responsible for general functions of the virus, whereas genus-specific and

species-specific genes are responsible for host interactions, and are likely to have been gained more recently (Davison et al., 2003). Current genomic organization of large-sized DNA viruses is the result of gene duplication, which may occur in all three types of genes, and lateral transfer of genes (Shackelton and Holmes, 2004). Lateral transfer of genes among homologous viruses co-infecting the same cell is an additional source of genes in large DNA viruses (Davison et al., 2003; Shackelton and Holmes, 2004).

Large population size, rapid replication, and host derived selection pressure resulting in genetic variation, are common observations with viruses (Parrish et al., 1991). DNA viruses are more species-specific with narrower host ranges than RNA viruses (Van Blerkom, 2003). Many DNA viruses are capable of integrating into the host genome, and stay in a metabolically inactive state known as latency, and this strategy enhances the fitness of the progeny viruses, thereby increasing the probability of transmission (Villarreal et al., 2000). This strategy results in chronic or latent infections with prolonged periods of infectivity, allowing these viruses to persist in small populations (Villarreal et al., 2000).

The utility of microbes for studying host history and demography depends on their mode of transmission (i.e. vertical, horizontal or both) (Ashford, 2000). Vertical transmission of viruses depends on host population structure and density (Villarreal et al., 2000). Vertically-transmitted pathogens are passed from parents to offspring within the host population. In a population, these pathogens are competent in revealing host population dynamics that reflect more ancient events (Kitchen et al., 2008). In contrast, horizontally-transmitted pathogens are able to transmit by direct or indirect contact among related and unrelated individuals within their host populations, and are likely to reveal more recent history and faster population dynamics of the host (Kitchen et al., 2008).

1.3.2. Virus evolution and molecular epidemiology

Molecular epidemiology utilizes molecular biological techniques to study the determinants of disease occurrence in populations (Foxman and Riley, 2001). Molecular techniques aid in disease surveillance, epidemiological investigations, identification of transmission patterns and risk-factors, characterization of host-pathogen interactions, detection of fastidious organisms and the study of molecular pathogenesis (Foxman and Riley, 2001). With the development of new molecular biological techniques including PCR-based methods and rapid sequencing of DNA, data on nucleotide sequences of various parts of the genomes of diverse organisms are accumulating at a faster rate (Duffy et al., 2008). Mutations of viruses, as well as the number and density of susceptible hosts in contact with viruses, contribute to their observed patterns of distribution (Holmes, 2004). Pathogen strains and phylogenic lineages are generated by mutation, and their survival depends on existing epidemiological and immunological forces (Grenfell et al., 2004).

Evolutionary relationships among genes and organisms are illustrated with phylogenetic trees (Vandamme, 2003). Single gene trees of large DNA viruses differ from one another due to lateral gene transfer, gene loss and gene duplication; hence, the evolutionary history of large DNA viruses are better represented by multiple phylogenies (Shackelton and Holmes, 2004). DNA viruses acquire genes from the host by gene transfer and this mechanism is used to escape from the host's immune selection. Therefore studies on whole genome analysis and detailed genome organization provide more accurate information on viral phylogenetic relationships (Shackelton and Holmes, 2004). Because of the lack of proof reading activity in RNA dependent RNA polymerase, RNA viruses are equipped with a high mutation rate which is used to escape

from immune pressure from the host (Holmes, 2004). In RNA viruses, phylogeny of an individual gene is likely to be a good representation of a whole genome (Holmes, 2004).

2.0. A SURVEY OF INFECTIOUS AGENTS IN MULE DEER (*Odocoileus hemionus*) AND WHITE-TAILED DEER (*Odocoileus virginianus*) IN SOUTHERN SASKATCHEWAN FOR THEIR POTENTIAL USE AS TOOLS TO EVALUATE MANAGEMENT STRATEGIES FOR THE CONTROL OF CHRONIC WASTING DISEASE

2.1. Abstract

As a part of an ongoing study of chronic wasting disease (CWD) in southern Saskatchewan, a survey was conducted during the winter seasons of 2006, 2007 and 2008 to determine the prevalence of CWD and other selected pathogens of mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*). Tonsil biopsies, feces and blood samples were collected from 254 wild mule deer and 43 wild white-tailed deer captured from five and three study locations, respectively. Immunohistochemical studies on tonsil biopsies for CWD revealed a 2.4% (6/249) prevalence in mule deer, while the disease was not detected in the white-tailed deer. Using fecal floatation, Baermann technique and fluorescent antibody test, the following taxa were identified in 253 mule deer fecal samples: nematodes of the superfamily: Trichostrongyloidea (29.2%); four genera of nematodes: *Nematodirus* (7.1%), *Skrjabinema* (14.2%), *Trichuris* (0.8%) and *Orthostrongylus* (35.2%); two genera of cestodes: *Moniezia* (16.2%) and *Thysanosoma* (11.9%); and two genera of protozoans: *Eimeria* (13.4%) and *Giardia* (0.7%). In 42 white-tailed deer, one superfamily of nematodes: Trichostrongyloidea (4.8%); one genus of nematodes: *Orthostrongylus* (2.4%); one genus of cestode: *Moniezia* (2.4%); and one genus of protozoa: *Eimeria* (2.4%); and dorsal-spined larvae (2.4%) were detected in feces of white-tailed deer. In mule deer, serum neutralization test and enzyme-linked immunosorbent assay revealed antibodies (Ab) against bovine herpesvirus 1(BoHV-1) (34.8%),

parainfluenza 3 (PI-3) (56.5%), bovine virus diarrhoea virus 1 (BVDV-1) (30.8%) and *Neospora caninum* (14.7%, 8/39)). In 40 white-tailed deer, serum Ab were detected against all four agents with a prevalence of 32.5% for BoHV-1, 35% for PI-3, 12.5% for BVDV-1, and 20.5% for *Neospora caninum*. Based on moderate prevalence, host specificity, direct or environmental modes of transmission herpesvirus, BVDV-1, PI-3, *Skrjabinema* and *Eimeria* were selected as potential tools to evaluate CWD management strategies.

2.2. Introduction

Chronic wasting disease (CWD) is a fatal, contagious, neurodegenerative disease of several cervid species in North America (Miller and Williams, 2004; Baeten et al., 2007). It is caused by CWD infectious prion protein (PrP^{CWD}) and is the only known transmissible spongiform encephalopathy (TSE) affecting free ranging species (Spraker et al., 1997; Williams and Miller, 2002). It is primarily reported in mule deer (*Odocoileus hemionus),* white-tailed deer (*Odocoileus virginianus),* elk (*Cervus elaphus*) and moose (*Alces alces*), and is likely to occur in subspecies of these cervid species (Williams et al., 2002a; Kahn et al., 2004). CWD has a long incubation period of approximately 23 months in mule deer and 12-34 months in elk under experimental conditions (Miller et al., 1998).

Direct contact and environmental contamination are the main modes of horizontal transmission of CWD (Spraker et al., 1997; Miller et al., 1998; Miller and Williams, 2003). The main sources of transmission are thought to be saliva, feces and urine (Spraker et al., 1997; Mathiason et al., 2006). Diagnosis of clinical CWD is based on observing microvacuolation and gliosis of the brain, in particular the medulla oblongata, in conjunction with the detection of the PrP^{CWD} by immunohistochemistry (IHC) (Williams et al., 2002a). Preclinical detection of infection is achieved by IHC examination of tonsil and lymph node biopsy (Miller et al., 1998;

Spraker et al., 2002a; Wild et al., 2002), or recto-anal mucosa associated lymphatic tissue (Wolfe et al., 2007). Tonsil biopsy is preferred to rectal biopsy because PrP^{CWD} deposits later in rectal mucosa than tonsillar follicles (Wolfe et al., 2007). Accurate negative diagnosis of CWD requires examination of a tonsil biopsy containing a minimum of 4 – 6 lymphoid follicles (Gavier-Widen et al., 2005).

In Canada, CWD was first reported in 1996 in a captive elk herd (Williams and Miller, 2002). Epidemiological investigations revealed the importation of an infected farmed elk from USA as the source of infection (Kahn et al., 2004; Dubé et al., 2006; Argue et al., 2007). In 2000, the disease was detected in free-ranging mule deer in Saskatchewan (Kahn et al., 2004). Since 1997, Saskatchewan Ministry of Environment and the Canadian Cooperative Wildlife Health Centre (CCWHC) have been engaged in passive surveillance for CWD using hunter-submitted deer heads (Williams and Miller, 2002). Presently, the disease in free-ranging cervids is found in three locations in Saskatchewan: along the South Saskatchewan River valley; within the boreal forest in an area approximately 100 km west of the Manitoba border; and along the Saskatchewan-Alberta border (Bollinger et al., 2008). Management of CWD is hampered by the long incubation period, subtle early clinical signs in infected individuals and limitations in detecting preclinical cases. It is further complicated by environmental contamination and extreme resistance of PrP^{CWD} to decontamination, along with an incomplete understanding on transmission of CWD (Williams and Miller, 2002; Williams et al., 2002a; Kahn et al., 2004).

Management strategies of CWD in endemic areas have focused on containment and reduction in prevalence through herd reduction, intensive culling, and bans on baiting (Williams and Miller, 2002). In Saskatchewan, CWD has been managed in free-ranging populations primarily by herd reduction (Bollinger et al., 2004). Early aggressive management through

selective culling or generalized population reduction have been shown to be important as management strategies (Williams et al., 2002a); however, early detection of infected populations is limited due to dependence on targeted or harvest-based surveillance programs (Gross and Miller, 2001). Further, the social behaviour of deer and elk, and large-scale migration in some populations have weakened the effectiveness of density reduction in controlling the disease (Williams and Miller, 2002).

Evaluation of the effectiveness of current management strategies is also challenging because of long incubation periods and relatively low overall prevalence (Williams et al., 2002a). Therefore, alternative methods of evaluating CWD management strategies are needed. Assessing the effectiveness of disease management strategies on other infectious agents which are transmitted in a manner similar to CWD, but which respond more rapidly to management actions, could be useful in predicting the eventual effect on management of CWD.

The objective of this study was to identify infectious agents which have the potential to respond rapidly to adopted management programs to control CWD in Saskatchewan. In order to find agents transmitted similarly to CWD through either close contact or environmental contamination, active surveillance was carried out in southern Saskatchewan for parasites, selected viruses and CWD. These surveillance activities were carried out during the winters of 2006, 2007 and 2008. Criteria for selecting an infectious agent as potential tools to evaluate the management strategies for the control of CWD includes similar modes of transmission (direct or environmental), host specificity and moderate prevalence. Bovine herpesvirus 1 (BoHV-1), parainfluenza 3 (PI-3) and bovine virus diarrhoea 1(BVD-1) were selected as infectious agents that are transmitted by close contact among cervids (Van Campen et al., 1997; Capman and

Early, 2001; King, 2001); and parasites of wild cervids were considered as agents that are transmitted through environmental contamination (Ezenwa, 2003).

2.3. Materials and methods

2.3.1. Serum samples, tonsil biopsies and fecal samples

Two hundred and fifty-four mule deer (*Odocoileus hemionus*) and forty-three white-tailed deer (*Odocoileus virginianus*) were captured by helicopter netting or clover trapping from Beechy community pasture (N50°50' to 51°08', W107°31', to 107°53'), Matador community pasture (N50°39' to 50°54', W107°26', to 108°02'NW), Douglas provincial park (N50°54' to 51°08', W106°13', to 106°39'NW), Swift Current Creek (N50°26' to 50°41', W107°34' to 107°58'NW) and Antelope Creek (N50°34' to 50°47', W108°03', to 108°26'NW) of southern Saskatchewan during the winters of 2006 (April), 2007(March to May) and 2008 (February to April) (Figure 1). These study locations were in a CWD endemic area.

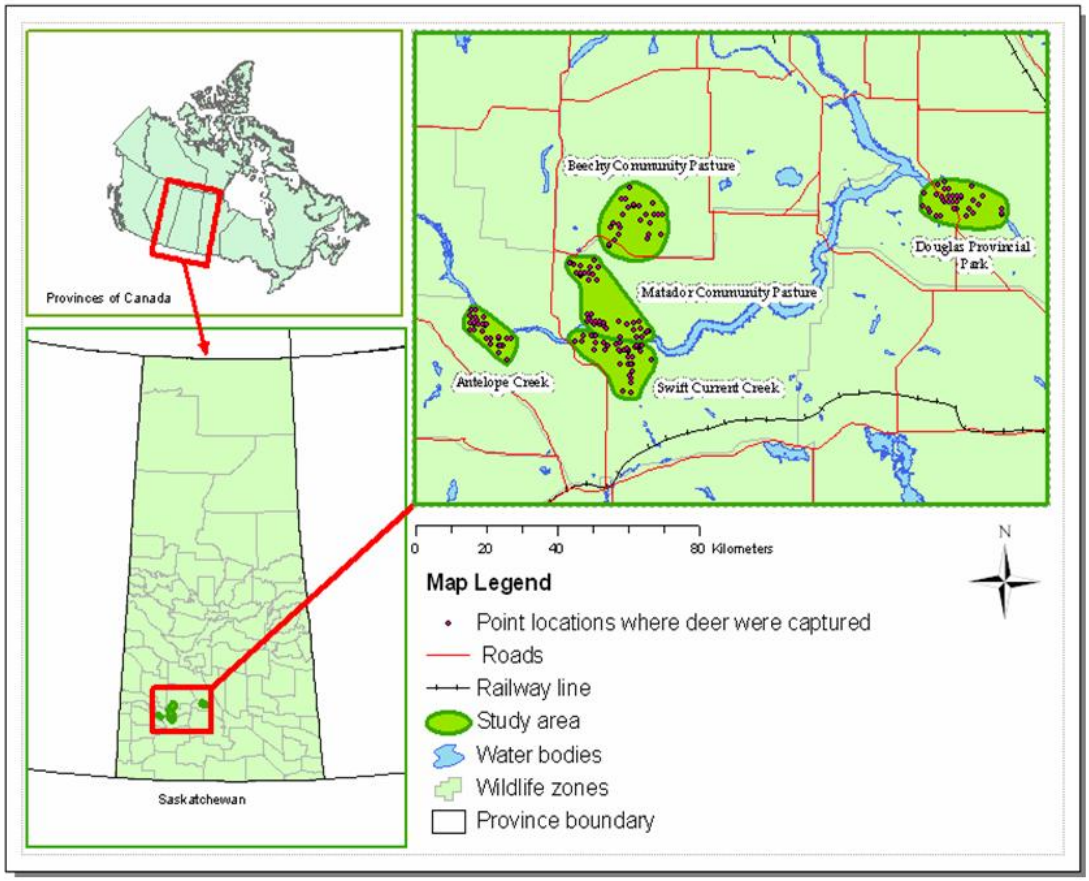

Figure 1. Study locations in southern Saskatchewan during winters of 2006, 2007 and 2008

Mule deer were sampled from all five study locations (Table 1) while white-tailed deer were sampled from three study locations (Table 2). In this study a deer ≤ 1 year of age was considered as a juvenile while those >1 year were considered as adults. Animal capture, handling and sampling protocols were approved by the University of Saskatchewan Animal Care Committee (permit #20050135).

Captured deer were anaesthetized by intramuscular injection of a combination of tiletamine hydrochloride and zolazepam hydrochloride (Telazol®, Fort Dodge, USA) and xylazine hydrochloride (Xylamax, Bimeda-MTC Animal Health, Canada). Blood samples, tonsil biopsies and fecal samples were collected from captured individuals and were ear-tagged, radio-collared, revived with atipamezole hydrochloride (Antisedan®, Pfizer Animal Health, PA), and released upon recovery. Blood for serum was collected into plain tubes (Vacutainer®, Becton Dickinson Labware, CA). Blood was also collected into heparinized tubes (Vacutainer ®, Becton Dickinson Labware, CA) to obtain buffy coat and plasma. Fecal samples were collected into sterile sample bags (VWR®, Canada), tonsil biopsies in 1.5 ml microcentrifuge tubes (VWR®, Canada), maintained at 4 °C until transported to the laboratory and stored at -20 °C. Blood was allowed to stand at 4 °C for 4 - 6 h., serum was separated by centrifugation at 1000 × g for 10 min. (Sorvall Instruments, CA), divided into aliquots, and stored at -80 °C. Heparinized blood tubes were centrifuged at 1500 × g for 10 min. (Sorvall Instruments, CA) at room temperature (25 °C) and red blood cells, buffy coat, and plasma fractions were separated and stored at -80 °C.

Table 1. Samples from mule deer collected for testing by location, age and sex in 2006, 2007 and 2008

	2006 (47)			2007 (164)				2008 (43)	
	Female (47)	Male (92)		Female (72)*		Male (26)		Female (17)	
	Adult	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
Antelope	0	20	10	12	10	3	3	1	0
Beechy	11	5	2	5	5	1	0	3	0
Douglas	12	9	7	5	12	2	1	3	3
Matador	12	10	12	0	8	6	2	4	1
Swift Current	12	12	5	10	4	6	2	2	0
Total	47	56	36	32	39	18	8	13	4

*age record was unavailable for one female mule deer in Swift Current Creek

Table 2. Samples from white-tailed deer collected for testing by location, age and sex in 2007 and 2008

	2007 (32)				2008 (11)			
	Male (14)		Female (18)		Male (3)		Female (8)	
	Adults	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
Douglas	3	0	3	0	0	2	4	1
Matador	6	1	8	2	1	0	2	1
Swift Current	2	2	4	1	0	0	0	0
Total	11	3	15	3	1	2	6	2

2.3.2. Immunohistochemical staining for CWD

Immunohistochemical staining of the tonsil biopsies of 249 mule deer and 43 white-tailed deer was done by Prairie Diagnostic Services Inc., Saskatoon, SK, Canada. Tonsil biopsies were trimmed to 2 - 3 mm thickness, placed in embedding cassettes and fixed in 10% neutral buffered formalin under agitation for 24 h. at 25 °C. Fixed tissues were immersed in 98% formic acid for 1 h., and then drained and washed in tap water to eliminate any remaining acid, and placed in fresh 10% neutral buffered formalin for 16 h. Then, tissues were processed using tissue processor (Histomatic 266, Fisher Scientific, Canada) and saturated with liquid paraffin. Tissues were embedded in liquid paraffin, sectioned at 5 µm sections, mounted on glass slides and incubated at 45 °C for 1 h. The excess paraffin on slides was removed by baking for 1 h. at 65 °C in a drying oven. Tissue sections were further deparaffinized with EZ Prep™ solution (Ventana Medical Systems Inc., AZ) and antigen retrieval was achieved by application of Ventana tissue conditioner CC-1 (Ventana Medical Systems Inc., AZ) followed by heating the slide at 95 °C for 8 min. The tissue sections were incubated at 100 °C for 82 min., while Ventana tissue conditioner CC-1 was applied every 4 min. Then, Ventana protease 3 (Ventana Medical Systems Inc., AZ) was applied onto the slide, incubated at 37 °C for 2 min. and washed with Ventana reaction buffer (Ventana Medical Systems Inc., AZ).

Slides were immunostained in batches of 20 slides which included two positive controls**.** An indirect immunostaining method was used to detect PrP^{res} in fixed tonsil biopsies. The slides were barcoded and placed in automated immunohistochemistry stainer (BenchMark™, Ventana Medical systems Inc., AZ), and incubated at 37 °C for 32 min. with primary monoclonal antibody to PrP^{res}, F99/97.6.1 (VMRD Inc., WA) diluted 1:1500 in Dako® antibody diluent (Dako Canada, Inc., Canada). Ventana reaction buffer **(**Ventana Medical Systems Inc., AZ) was

then used to wash the slides**.** Biotinylated secondary antibody (Ventana enhanced V-red detection kit, Ventana Medical Systems Inc., AZ) was then applied to the slide and incubated for 8 min., alkaline phosphatase-conjugated streptavidin was applied and incubated for 12 min. and fast red napthol based chromogen was applied and incubated for 20 min. Slides were washed with Ventana reaction buffer after each incubation step**.** The slides were counterstained with Ventana Nexes Hematoxylin (Ventana Medical Systems, AZ) and Ventana bluing reagent (Ventana Medical Systems, AZ), each for 6 min. The slides were washed in 5% dish washing soap (Dawn®, Proctor and Gamble, OH), rinsed in running tap water, dehydrated through graded alcohol series, cleared in xylene and coverslipped using a xylene-based embedding agent (Entellan®, Electron microscopy Sciences, PA). Tissue sections were examined under light microscope for the presence of characteristic red staining on sections. Tissue sections on slides that were diagnosed as positive, or those that showed high levels of background staining, were treated with a second formic acid treatment, proteinase-K digestion, and hydrated autoclaving to confirm the initial diagnosis. In the tonsil biopsy sections, lymphoid follicles that had red chromogen deposits were interpreted as positive for CWD, while a minimum of six follicles were examined to confirm the section as negative (Gavier-Widen et al., 2005). Therefore the biopsies which were negative but consisted of < 6 follicles were interpreted as inconclusive.

2.3.3. Serum neutralization test for BVDV-1, BoHV-1 and PI-3

Serum neutralization tests (SNT) on serum samples of 253 mule deer and 40 white-tailed deer were performed by Prairie Diagnostic Services Inc., Saskatoon, SK, Canada. Serum samples were heat inactivated at 56 °C for 30 min. Four three-fold serial dilutions of serum in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corporation, Canada), supplemented with 5% fetal bovine serum (FBS) (Invitrogen Corporation, Canada), penicillin G 100 IU/ml

(Invitrogen Corporation, Canada), streptomycin 100 µg/ml (Invitrogen Corporation, Canada), gentamicin 100 µg/ml (Invitrogen Corporation, Canada) and enrofloxacin 1 µg/ml (Invitrogen Corporation, Canada), were placed in duplicate in 96-well tissue culture plates (BD Falcon™ 96-multiwell plate, Becton Dickinson Labware, CA). Positive and negative control sera were also included in each assay (positive control serum for BoHV-1 was obtained from cattle vaccinated against infectious bovine rhinotrachitis (IBR) and a serum sample which was found to be negative for BoHV-1 was used as the negative control). One hundred 50% tissue culture infective dose ($TCID_{50}$) of BoHV-1, strain P8-2 (kindly provided by Dr J.R. Saunders, University of Saskatchewan); BVDV-1, Singer strain; or PI-3, SF-4 strain (American Type Culture Collection, VR-281, VA) were added to each well and incubated at 37 °C for 2 h. A cell suspension prepared from embryonic bovine tracheal cells in DMEM supplemented with 5% FBS serum and antibiotics (penicillin G 100 IU/ml, streptomycin 100 µg/ml, gentamicin 100 µg/ml and enrofloxacin 1 µg/ml) was then added, sealed, and incubated at 37 °C in 5% CO_2 and 85% humidity for 7 days. After seven days, plates were observed for the presence or absence of cytopathic effects using an inverted microscope (Olympus CK-2, Baxter / Canlab Ltd., Canada). Serum end-point titre was defined as the reciprocal of the serum dilution that inhibited the cytopathic effects of the virus. The suggested guidelines provided by the diagnostic laboratory were: SNT titres <6 was negative , or SNT titres were ≥6 was positive (Waldner, 2005),

2.3.4. *Neospora caninum* enzyme-linked immunosorbent assay

Serum samples from 245 mule deer and 39 white-tailed deer were tested for Ab against *Neospora caninum* using a commercial competitive ELISA kit, (*Neospora caninum* antibody test kit, cELISA, VMRD Inc., WA) and the manufacturer recommended protocols. Briefly, 50 µl of serum from each sample, along with controls, were added in duplicate to antigen-coated plates

and incubated for 1 h. at 25 °C. Fifty microliters of 1× antibody-peroxidase conjugate was added to each well and incubated for 20 min. at 25 °C and 50 µl of the substrate solution was added and incubated for 20 min. at 25 °C. Between each step, plates were washed three times with washing buffer. The reaction was stopped by adding 50 µl of stop solution. The optical density (OD) was measured at 650 nm wavelength using an ELISA reader (SPECTRAmax, Molecular Devices Corporation, CA). Percentage inhibition (PI) was calculated by the following formula:

$$PI = 100 - [(\text{sample}_{OD} \times 100) / (OP\ (\text{mean negative control}_{OD})].$$

According to manufacturer recommendations, the results were interpreted as positive if inhibition was ≥30%, or negative if inhibition was <30%. This was based on the validation to test cattle serum (Baszler et al., 1996).

2.3.5. Detection of nematode eggs, cestode eggs and protozoan oocysts in feces

Fecal samples from 253 mule deer and 42 white-tailed deer were analysed by Wisconsin double centrifuge technique (Cox and Todd, 1962). Briefly, 5 g of feces were collected into a paper cup, soaked for 10 min. in 12 ml of water, and mixed with a spatula. The paper cup was squeezed to make a spout and fecal suspension was passed through a single layer of cheese-cloth into a second paper cup. The filtrate was transferred into a labelled 16×125 mm test tube and centrifuged for 10 min. at 200 × g (Damon IEC Model HN-S, Thermo Fisher Scientific, MA), and supernatant was decanted without disturbing the sediment in a single pouring motion. The sediment was resuspended in 5 ml of Sheather's solution (454 grams of table sugar, 355 ml water and 6 ml 40% formaldehyde), mixed and topped with Sheather's solution until a slightly convex fluid meniscus was formed. A 22 × 22 mm cover slip was carefully placed on the meniscus and the test tube was centrifuged at 200 × g for 10 min., the cover slip was then gently

lifted straight up and transferred onto a glass slide, and examined under a light microscope (Nikon Labophot-2, Nikon Corporation, Japan) at 100 × total magnification. Parasite eggs and oocysts were identified based on morphology (Foreyt and Foreyt, 2001), counted and recorded as eggs per gram (epg) or oocysts per gram (opg) of feces. The epg or opg values were used to calculate mean intensity and range.

2.3.6. Recovery of protostrongylid larvae from feces

Parasitic larvae were identified and quantified from feces of 253 mule deer and 42 white-tailed deer with a modified beaker Baermann technique (Forrester and Lankester, 1997; Jenkins et al., 2005). Five grams of fecal pellets were placed in the pocket of an envelope lined by two 7×6 cm sized, 2×2 mm nylon meshes separated by cellulose tissue (Kimwipes®, Kimberly-Clark Corporation, WI). Then, the envelope was submerged horizontally with the pocket facing upwards in a beaker containing 200 ml lukewarm water and allowed to stand for 24 h., after which the envelope was discarded and the filtrate allowed settling for 5 min. Upon settling, the beaker contents were drawn off from the top by siphon until 30 ml (approximately) was retained. The suspension was then fractioned into two 16 × 125 mm test tubes, along with 2 ml of water used to rinse the beaker. Both tubes were centrifuged at 200 × g for 10 min. The fluid was then siphoned from each tube at the meniscus until 2 ml (approximately) was retained, and the retained contents of one tube were transferred to the other, and centrifuged at 200 × g for 10 min. The fluid was again siphoned out at the meniscus until 2 ml (approximately) was retained. The retained fluid (approximately 2 ml) was placed on glass slides and coverslipped, and examined under a light microscope (Nikon Labophot-2, Nikon Corporation, Japan) at 40 × magnification. Larvae were identified on tail morphology (Foreyt and Foreyt, 2001), counted and recorded as

larvae per gram (lpg) of feces. The lpg values were used to calculate the mean intensity and the range.

2.3.7. Detection of trematode eggs in feces

The detection of trematode eggs from 91 mule deer and 16 white-tailed deer was done by differential filtration followed by differential sedimentation using a specialized apparatus (FLUKEFINDER®, Soda Springs, ID, USA). Briefly, 2 grams of feces was suspended in 30 ml of water, poured into the top section of the FLUKEFINDER®, and allowed to pass through the screens. The FLUKEFINDER® was then half-filled with water and the passage of water through the screens was again facilitated; this step was repeated another three times. The top part of the FLUKEFINDER® was placed on a paper cup and the screen was back-washed with a stream of water using a squirt bottle. The suspension was swirled and poured into a 16 × 125 mm test tube and allowed to settle for 4 min. The supernatant was decanted in one pouring motion and the tube was half re-filled with water and allowed to settle for 2 min. Decanting the supernatant and half re-filling the test tube was repeated another 3 times. Finally, the supernatant was poured off and the sediment was swirled and poured onto a Petri dish, stained with methylene blue, and observed under a 25 × magnification of a dissecting microscope (Leica MZ6, Germany).

2.3.8. Fluorescent antibody test for the detection of *Giardia* and *Cryptosporidium* species

A fluorescent antibody test kit (Cryst-a-Glo™, WATERBORNE™, Inc., New Orleans, LA) was used to detect the cyst and oocyst stages of both *Giardia* and *Cryptosporidium* species from fecal samples collected from 138 mule deer and 24 white-tailed deer. Briefly, 2 grams of feces was suspended in 0.5 ml of water, and a thin fecal smear was prepared and air dried. A

drop of Cryst-a-Glo™ antibody was added onto the smear and incubated in a humid chamber at 37 °C for 30 min. A drop of counter stain was added to the smear, which was then air dried, mounted with a drop of mounting medium, cover slipped and observed under fluorescence microscope (Nikon Labophot-2, Nihon Corporation, Japan). Positive controls for *Giardia* and *Cryptosporidium,* supplied with the kit, were used with each batch of samples. According to the manufacturer's protocol, *Giardia* appear as oval-shaped cysts and *Cryptosporidium* as round cysts. Results were recorded as positive if green fluorescence was observed.

2.3.9. Statistical analysis

The unconditional associations between the primary outcome of interest CWD status based on tonsil biopsy and the potential risk factors, age class, sex and year of observation and location were examined using binary logistic regression (SPSS 16.0 for Windows®, SPSS Inc. Chicago, IL). Similarly unconditional associations between the same group of risk factors and the prevalence of serum Ab against BoHV-1, BVDV-1, PI-3 and *Neospora caninum*, as well as evidence of fecal shedding of various parasites (Trichostrongyloidea, *Nematodirus, Skrjabinema, Trichuris, Orthostrongylus*, dorsal spinal larvae, *Moniezia*, *Thysonasoma*, *Eimeria* and *Giardia*) were examined. The comparison of sex, age-class and locations were done with the samples collected during 2007 and 2008 which included 206 mule deer and 43 white-tailed deer. Adult female mule deer fraction is used for the comparison among years which includes 48 individuals. For each outcome of interest, all risk factors where $p < 0.20$ were identified for consideration in building final multivariable models. All variables that were statistically significant ($p < 0.05$) or potential confounders, defined as variables whose inclusion in the model changed the coefficients of other risk factors of interest by more than 10%, were retained in the model. Where two or more variables were statistically significant, biologically reasonable

interaction terms were examined and were retained in the model if the interaction term was statistically significant. Associations were reported as odds ratio (OR) with 95% confidence interval (95% CI).

2.4. Results

2.4.1. Chronic wasting disease

Chronic wasting disease was detected in 2.4% (6/249) of mule deer. In mule deer, 84.3% (210/249) were negative for CWD and 13.3% (33/249) were inconclusive. There was no significant difference in prevalence of CWD among study areas ($p = 0.8$). However, CWD was detected only in adult mule deer in the Antelope Creek (5/36, 13.9%) and Swift Current Creek (1/40, 2.5%) study areas. As Antelope Creek had the highest prevalence, it was used for detailed analysis. Once the 2 inconclusive CWD status samples in each age class were excluded, no significant difference in prevalence of CWD was detected between adults (5/34, 14.7%) and juveniles (0/21, 0%) ($p = 0.08$); between adult males (4/22, 18.2 %) and adult females (1/12, 8.3%) ($p = 0.45$); or between the years 2007 (5/48, 10.4%) and 2008 (0/7, %) ($p = 0.49$), in Antelope Creek. Chronic wasting disease was not detected in white-tailed deer 0% (34/43); CWD status was inconclusive in 20.9% (9/43) of the samples.

2.4.2. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3 (PI-3), bovine virus diarrhoea (BVDV-1) and *Neospora caninum* in mule deer

The overall prevalence of Ab to BoHV-1, BVDV-1, PI-3 and *Neospora caninum* were 34.8 % (88/253), 30.8% (78/253), 56.5% (143/253) and 15.4% (36/245), respectively. The adult age class had a significantly higher prevalence of Ab titres to viral agents compared to juveniles: 46.6% (55/118) of adults had Ab titres to BoHV-1 compared to 4.5% (4/88) in juveniles ($p <$

0.0001) when controlled for sex; 44.1 % (52/118) of adults had Ab titres to BVDV-1 compared to 8% (7/88) in juveniles ($p < 0.0001$); and 72% (85/118) of adults had Ab titres to PI-3 compared to 29.5% (26/88) in juveniles ($p < 0.0001$) when controlled for location. No significant difference in the prevalence of Ab against *Neospora caninum* was detected between adults (21/114, 18.4%) and juveniles (9/85, 10.6%) ($p = 0.23$) when controlled for sex. Based on the OR, adults are more likely to be exposed to BoHV-1(OR = 23, 95% CI = 8.1 - 67), PI-3 (OR = 17.5, 95% CI = 7.4 – 41) and BVDV-1 (OR = 8.8, 95% CI = 3.8 - 20) than juveniles.

In the comparison of sex classes, there was a significant difference in prevalence of serum Ab against BoHV-1 between females (29/91, 31.9%) and males (30/115, 26.1%) ($p = 0.006$) when controlled for age; and no significant difference in prevalence of serum Ab against BVDV-1 between females (25/91, 27.5%) and males (34/115, 29.6%) ($p = 0.78$) when controlled for age; PI-3 between females (48/91, 52.7%) and males (63/115, 54.8%) ($p = 0.19$) when controlled for age and location; and *Neospora caninum* between females (15/88, 17%) and males (15/111, 13.5%) ($p = 0.75$) when controlled for age. Based on OR males are more likely to be exposed to BoHV-1(OR = 2.3, 95% CI = 1.3 - 4.3) than females. Prevalence in 2006, 2007 and 2008 winters were compared only for adult female mule deer, and no significant differences were observed for BoHV-1($p = 0.82$) when controlled for age and sex, PI-3($p = 0.42$) when controlled for age and location, BVDV-1($p = 0.16$) when controlled for age and *Neospora caninum* ($p = 0.52$) when corrected for age and sex (Figure 2).

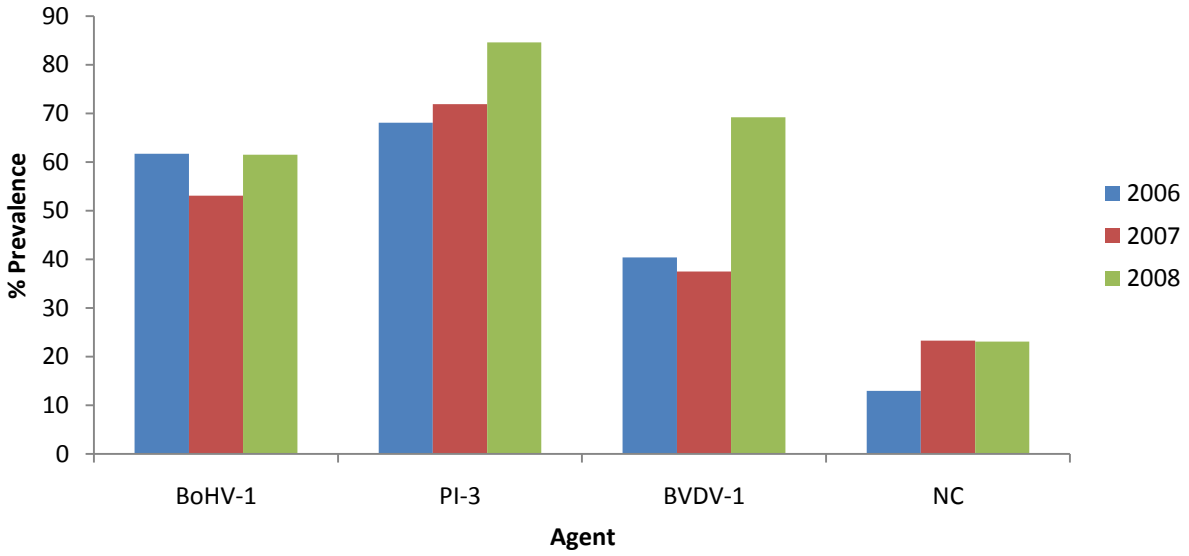

Figure 2. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3 (PI-3), bovine virus diarrhoea virus 1 (BVD-1) and *Neospora caninum* (NC) in adult female mule deer in winters of 2006, 2007 and 2008.

Serum Ab against BoHV-1, BVDV-1, PI-3 and *Neospora caninum* were detected in all locations (Figure 3, Table 3). Significant differences in prevalence were observed among locations for exposure to PI-3 ($p = 0.0001$) when controlled for age; BVDV-1 ($p = 0.0001$) when controlled for age; and no significant difference was detected against BoHV-1 ($p = 0.77$)) when controlled for sex and age; *Neospora caninum* ($p = 0.85$) when controlled for age and sex. Summary of the contrast analysis of PI-3 and BVD-1 between locations is shown in Table 4.

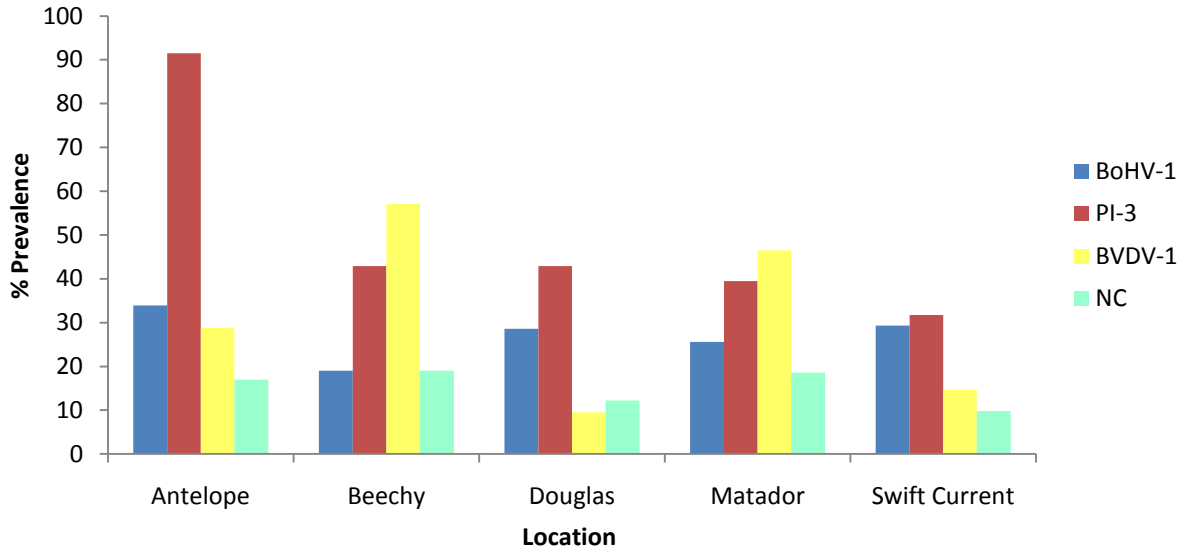

Figure 3. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3(PI-3), bovine virus diarrhoea virus 1 (BVDV-1) and *Neospora caninum* (NC) in mule deer from Antelope Creek, Beechy community pasture,Douglas provincial park, Matador community pasture and Swift Current Creek in winters of 2007 and 2008.

Table 3. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3(PI-3), bovine virus diarrhoea virus 1 (BVDV-1) and *Neospora caninum* (NC) in mule deer from Antelope Creek, Beechy community pasture,Douglas provincial park, Matador community pasture and Swift Current Creek in winters of 2007 and 2008

Location	BoHV-1	PI-3	BVDV-1	*Neospora caninum*
Antelope Creek	20/59 (33.9%)	54/59 (91.5%)	17/59 (28.8%)	9/53 (17%)
Beechy	4/21 (19%)	9/21(42.9%)	12/21 (57.1%)	4/21 (19%)
Douglas	12/42 (28.6%)	18/42 (42.9%)	4/42 (9.5%)	5/41 (12.2%)
Matador	11/43 (25.6%)	17/43 (39.5%)	20/43 (46.5%)	8/43 (18.6%)
Swift Current	12/41 (29.3%)	13/41(31.7%)	6/41 (14.6%)	4/41 (9.8%)

Table 4. Summary of contrast analysis on study locations in winters of 2007 and 2008 in mule deer against BoHV-1, PI-3 and BVDV-1

Contrasts of Location	PI-3	BVDV-1
Beechy verses Antelope	OR = 42 95% CI = 5-354 $p = 0.001$	OR = 0.27 95% CI = 0.07-0.78 $p = 0.02$
Beechy verses Matador	$p = 0.59$	$p = 0.89$
Beechy verses Douglas	$p = 0.79$	OR=0.08 95% CI = 0.02-0.39 $p = 0.002$
Douglas verses Antelope	OR = 16 95% CI=4.8-53 $p < 0.0001$	$p = 0.13$
Douglas verses Matador	$p = 0.32$	OR=12.6 95% CI = 3.1 - 51 $p<0.0001$
Matador verses Antelope	OR = 28 95% CI = 7.8-100 $p < 0.0001$	OR = 0.21 95% CI = 0.07-0.59 $p = 0.003$
Swift Current verses Antelope	OR = 86 95% CI = 22-331 $p < 0.0001$	OR = 3.2 95% CI = 1.1-10 $p = 0.03$
Swift Current verses Beechy	$p = 0.06$	OR = 14.5 95% CI=3.6-50 $p < 0.0001$
Swift Current verses Douglas	OR = 4.8 95% CI = 1.8-12 p=0-002	*p = 0.79*
Swift Current verses Matador	OR = 2.9 95% CI = 1.2-7.2 p = 0-04	OR =17.5 95% CI=4.4-100 $p < 0.0001$

2.4.3. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), para-influenza 3(PI-3), bovine virus diarrhoea (BVDV-1) and *Neospora caninum* in white-tailed deer in winters of 2007 and 2008

The overall prevalence of antibodies to BoHV-1, BVDV-1, PI-3 and *Neospora caninum* were 32.5% (13/40), 12.5% (5/40), 35% (14/40) and 20.5% (8/40), respectively. When age-classes were compared, there was no significant difference in prevalence of Ab titres to BoHV-1 between adults (12/30, 40%) and juveniles (1/10, 10%) ($p = 0.26$) when controlled for location and year; PI-3 between adults (13/30, 43.3%) and juveniles (1/10, 10%) ($p = 0.08$); BVDV-1 between adults (5/30, 16.7%) and juveniles (0/10, 0) ($p = 0.99$); and *Neospora caninum* between adults (7/29, 24.1%) and juveniles (1/10, 10%) ($p = 0.59$) when controlled for year and location. When comparing sex-classes, there was no significant difference in prevalence of Ab titres to BoHV-1 between females (7/26, 26.9%) and males (6/14, 42.9%) ($p = 0.31$) when controlled for location and year; to PI-3 between females (10/26, 38.5%) and males (4/14, 28.6%) ($p = 0.76$) when controlled for age; to BVDV-1 between females (3/26, 11.5%) and males (2/14, 14.3%) ($p = 0.8$), and to *Neospora caninum* between females (6/26, 23.1%) and males (2/13, 15.4%) ($p = 0.24$) when controlled for year and location. Similar results were found upon testing for differences between years. There was no significant difference in prevalence of BoHV-1 between 2007 (12/29, 41.4%) and 2008 (1/11, 9.1%) ($p = 0.47$) when controlled for location; PI-3 between 2007 (10/29, 34.5%) and 2008 (4/11, 36.4 %) ($p = 0.64$) when controlled for age; BVDV-1 between 2007 (3/29, 10.3%) and 2008 (2/11, 18.2%) ($p = 0.5$); and *Neospora caninum* between 2007 (8/28, 28.6 %) and 2008 (0/11, 0%) ($p = 0. 99$) when controlled for location. Differences in prevalence of Ab titres to these agents were observed among locations. Serum Ab against BoHV-1 and PI-3 and *Neospora caninum* was observed in all three locations, whereas

Ab against BVDV-1 was found only in Matador community pasture and Douglas provincial park (Figure 4, Table 4). No significant differences in prevalence were observed among locations for exposure to PI-3 ($p = 0.43$) when controlled for age; BVDV-1 ($p = 0.58$); BoHV-1 ($p = 0.58$)) when controlled for year; *Neospora caninum* ($p = 0.31$) when controlled for year.

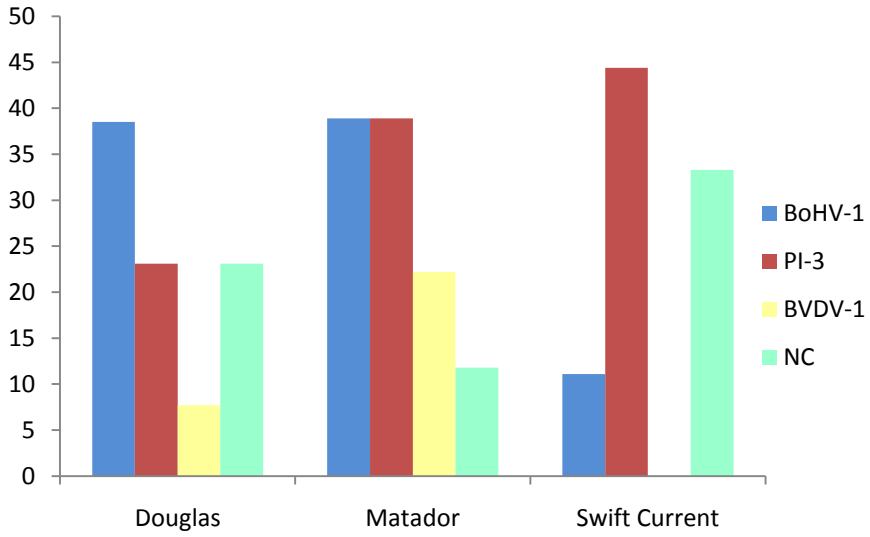

Figure 4. Prevalence of antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3(PI-3), bovine virus diarrhoea (BVDV-1) and *Neospora caninum* (NC) in white-tailed deer from Douglas provincial park, Matador community pasture and Swift Current Creek in winters of 2007 and 2008

Table 4. Prevalence of serum antibodies to bovine herpesvirus 1(BoHV-1), parainfluenza 3(PI-3), bovine virus diarrhoea (BVDV-1) and *Neospora caninum* (NC) in white-tailed deer from Douglas provincial park, Matador community pasture and Swift Current Creek in winters of 2007 and 2008

Location	Douglas	Matador	Swift Current Creek
BoHV-1	5/13 (38.5%)	7/18 (38.9%)	1/9 (11.1%)
BVDV-1	1/13 (7.7%)	4/18 (22.2%)	0/9 (0%)
PI-3	3/13 (23.1%)	7/18 (38.9%)	4/9 (34.4%)
Neospora caninum	3/13 (23.1%)	2/17 (11.8%)	3/9 (33.3%)

2.4.4. Parasitological studies

2.4.4.1. Parasitological studies of mule deer

Eggs, larvae or oocysts of one superfamily of nematodes, and eight genera of parasites were detected in fecal samples. Helminths included one superfamily of nematodes (Trichostrongyloidea), four genera of nematodes (*Nematodirus, Skrjabinema, Trichuris, and Orthostrongylus*), two genera of cestodes (*Moniezia* and *Thysanosoma*) and two genera of protozoa (*Eimeria* and *Giardia*). The overall prevalence of different parasites is given in Table 5. The most prevalent parasitic genera in mule deer were *Orthostrongylus*. Parasitic genera with a prevalence of > 1% were used for detailed analysis.

Table 5. Overall prevalence, mean intensity and range of fecal parasite eggs, larvae and oocysts in 253 mule deer in southern Saskatchewan from winter 2006, 2007 and 2008.

Parasite	Prevalence (%)	Mean	
		Intensity	Range
Nematoda			
Trichostrongyloidea	29.2 (74/253)	0.7 (51.4/74)	0.2 - 6.2
Nematodirus	7.1 (18/253)	1.3 (25/18)	0.2 - 4.2
Orthostrongylus	35.2 (89/253)	17.5 (1560.3/89)	0.2 - 205.5
Skrjabinema	14.2 (36/253)	3.4 (124.8/36)	0.2 - 31
Trichuris	0.8 (2/253)	0.4 (0.8/2)	0.2 - 0.6
Cestoda			
Moniezia	16.2 (41/253)	28 (1149.9/41)	0.2 - 241.4
Thysanosoma	12.2 (31/253)	3.3 (103.65/31)	0.2 - 33.2
Protozoa			
Eimeria	13.4 (34/253)	7.5 (253.9/34)	0.2 - 153
Giardia	0.7 (1/138)	NA	NA
Cryptosporidium	0 (0/138)	NA	NA
Trematodes	0 (0/91)	NA	NA

NA = not applicable

Different age classes were compared, and there was a significant difference in prevalence of *Nematodirus* between adults (2/118, 1.7%) and juveniles (16/87, 18.4%) ($p < 0.0001$) when controlled for location and sex; *Moniezia* between adults (10/118, 8.5%) and juveniles (28/87, 32.2%) ($p < 0.0001$) when controlled for location; *Thysanosoma* between adults (6/118, 5.1%) and juveniles (22/87, 25.3%) ($p < 0.0001$); and *Orthostrongylus* between adults (37/118, 31.4%) and juveniles (47 /87, 54%) ($p < 0.0001$) controlled for year and sex; however, no significant difference was found in prevalence of Trichostrongyloidea between adults (31/118, 26.3%) and juveniles (29/87, 33.3%) ($p = 0.27$), *Skrjabinema* between adults (15/118,12.7%) and juveniles (16/87, 18.4%) ($p = 0.26$), and *Eimeria* between adults (18/118,15.3%) and juveniles (15/87, 17.2%) ($p = 0.70$). Based on OR, juveniles have higher risk of shedding parasitic stages of *Nematodirus* (OR = 4.4, 95% CI = 2-10), *Moniezia* (OR = 4.6, 95% CI = 2-10), *Orthostrongylus* (OR=2.6, 95% CI = 1.4 – 4.8) and *Thysanosoma* (OR = 6.3, 95% CI = 2.4 - 16) than adults.

Within sex-classes, no significant difference in prevalence of *Orthostrongylus* between females (31/91, 34.1%) and males (53/115, 46.1%) ($p = 0.11$), *Nematodirus* between females (12/89, 13.5%) and males (6/117, 5.1%) was detected ($p = 0.18$) when controlled for age and location, Trichostrongyloidea between females (27/89, 30.3%) and males (34/117, 29.1%) ($p = 0.89$), *Skrjabinema* between females (15/89, 16.9%) and males (16/117, 13.7%) ($p = 0.53$), *Moniezia* between females (19/89, 21.3%) and males (19/117, 16.2%) ($p = 0.35$), *Thysanosoma* between females (12/89, 13.5%) and males (16/117, 13.7%) ($p = 0.96$) and *Eimeria* between females (12/89, 13.5%) and males (21/117, 17.9%) ($p = 0.38$) was observed.

Differences in the prevalence of parasite fecal shedding among locations were observed (Figure 5, Table 6). Parasitic eggs or oocysts of Trichostrongyloidea, *Skrjabinema*, *Protostrongylus, Moniezia* and *Eimeria* were detected in all study locations, and were considered

in detailed analysis. Among the study locations, a significant difference in prevalence was observed for Trichostrongyloidea ($p < 0.0001$) when controlled for sex and year. No significant difference in prevalence among study locations was detected for *Moniezia* ($p = 0.54$) when controlled for age; *Eimeria* ($p = 0.24$) when controlled for year, sex and age; *Orthostrongylus* ($p = 0.49$) when controlled for age, year and sex; and *Skrjabinema* ($p = 0.12$) controlled for year. Summary of the contrast analysis of Trichostrongyloidea prevalence among locations is shown in Table 7.

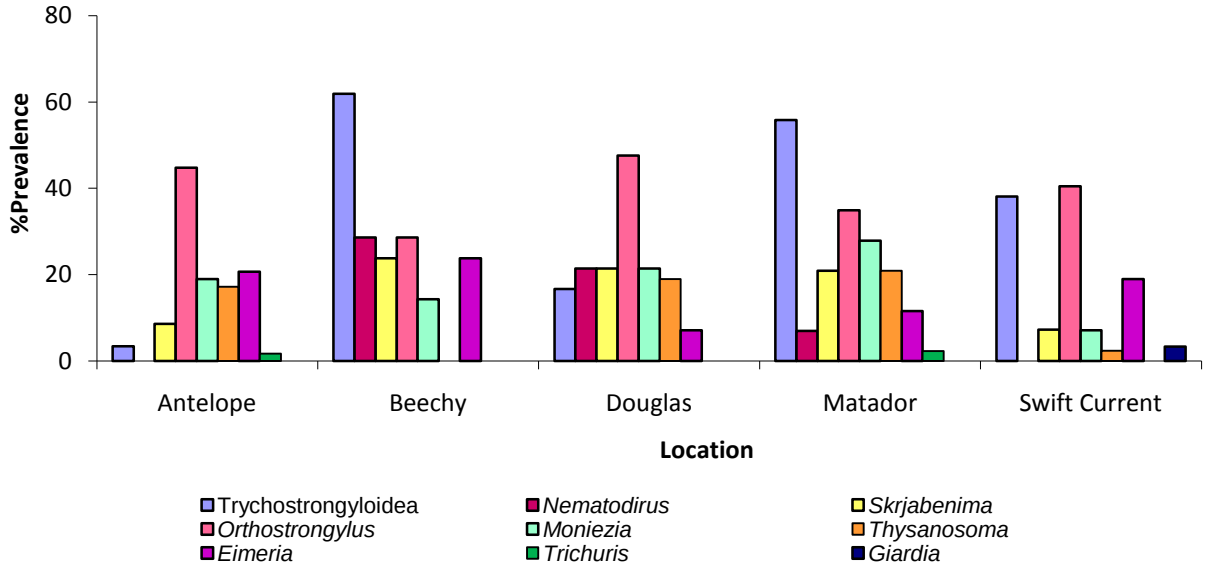

Figure 5. Comparison of the prevalence of parasites in mule deer among locations in winters of 2007 and 2008

Table 6 prevalence of parasites in mule deer among locations in winters of 2007 and 2008

Location	Antelope	Beechy	Douglas	Matador	Swift Current
Trichostrongyloidea	2/58 (3.4%)	13/21 (61.9%)	7/42 (16.7%)	24/43 (55.8%)	15/42 (37.55)
Nematodirus	0/58 (0%)	6/21 (28.6%)	9/42 (21.4%)	3/43 (7%)	0/42 (0%)
Orthostrongylus	26/58 (44.8%)	6/21 (28.6%)	20/42 (47.6%)	15/43 (34.9%)	17/42 (40.5%)
Skrjabinema	5/58 (8.6%)	5/21 (23.8%)	9/42 (21.4%)	9/43 (20.9%)	3/42 (7.1%)
Trichuris	1/58 (1.7%)	0/21 (0%)	0/42 (0%)	1/43 (2.3%)	0/42 (0%)
Moniezia	11/58 (19%)	3/21 (14.3%)	9/42 (21.4%)	12/43 (27.9%)	3/42 (7.1%)
Thysanosoma	10/58 (17.2%)	0/21 (0%)	8/42 (19%)	9/43 (20.9%)	1/42 (2.4%)
Eimeria	12/58 (20.7%)	5/21 (23.8%)	3/42 (7.1%)	5/43 (11.6%)	8/42 (19%)
Giardia	0/48 (0%)	0/17 (0%)	0/32 (0%)	0/10 (0%)	1/31 (3.2%)
Cryptosporidium	0/48 (0%)	0/17 (0%)	0/32 (0%)	0/10 (0%)	0/31 (0%)
Trematodes	0/13 (0%)	0/12 (0%)	0/24 (0%)	0/21 (0%)	0/20 (0%)

Table 7. Summary of contrast analysis among locations in 2007 and 2008 for Trichostrongyloidea

Contrasts of Location	Trichostrongyloidea
Antelope verses Beechy	OR = 52, 95% CI = 9.6 - 286; $p < 0.0001$
Antelope verses Douglas	OR = 6, 95% CI = 1.2 - 31; $p = 0.03$
Antelope verses Matador	OR = 49, 95% CI= 10 - 237; $p < 0.0001$
Antelope verses Swift Current	OR = 18.3, 95% CI = 3.8 - 87; $p < 0.0001$
Beechy verses Matador	$p = 0.90$
Douglas verses Beechy	OR = 8.6, 95% CI = 2.5 - 29 ; $p = 0.001$
Douglas verses Matador	OR = 7.4, 95% CI = 2.5-21; $p < 0.0001$
Douglas verses Swift Current	OR = 2.9, 95% CI = 1 – 8.3; $p = 0.043$
Swift Current verses Beechy	$p = 0.6$
Swift Current verses Matador	OR = 2.6, 95% CI = 1 - 6.3; $p = 0.03$

Parasitological prevalences among years were compared only for adult female mule deer (Figure 6). As eggs of the superfamily of nematodes Trichostrongyloidea; and *Skrjabinema, Moniezia*, larvae of *Orthostrongylus*, and oocysts of *Eimeria* were detected in 2006, 2007 and 2008, they were used in the detailed analysis. No significant difference was observed among 2006, 2007 and 2008 for Trichostrongyloidea ($p = 0.13$) controlled for location, *Skrjabinema* ($p = 0.16$) controlled for location, *Moniezia* ($p = 0.96$) when controlled for location and *Eimeria* oocysts ($p = 0.51$) when controlled for location, nor was a difference among years observed for *Orthostrongylus* larvae ($p = 0.09$).

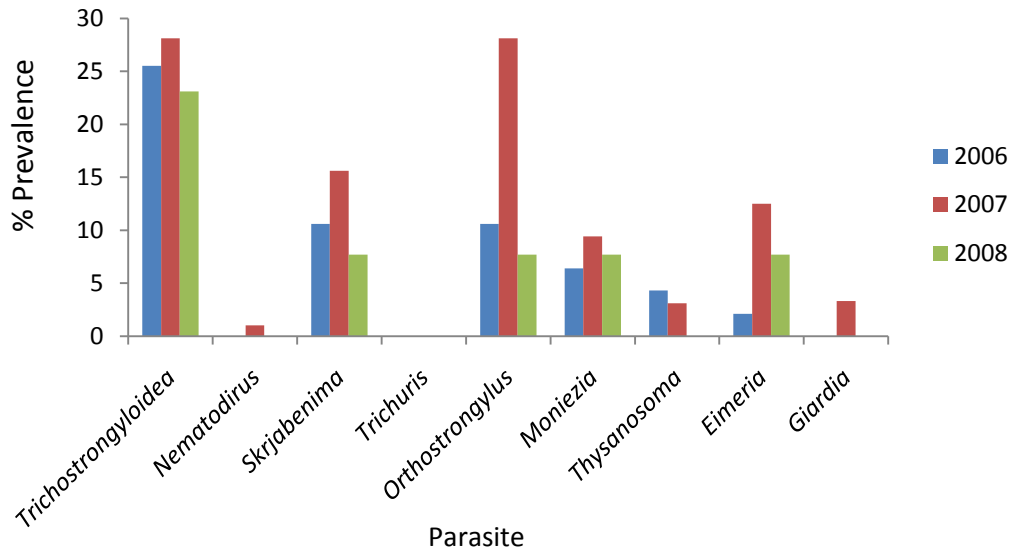

Figure 6. Parasitological prevalence among years in adult female mule deer in winters of 2006, 2007 and 2008

2.4.4.2. Parasitological studies of white-tailed deer

Eggs, larvae and oocysts of five parasitic genera were detected in fecal samples. A super family of nematodes (Trichostrongyloidea), two genera of nematodes (*Orthostrongylus* and dorsal-spined larvae) were detected, along with one genus of cestode (*Moniezia*) and one genus of protozoa (*Eimeria*). The overall prevalence, mean intensity and range are given in Table 7. In comparisons of age-classes, no significant difference in prevalence of Trichostrongyloidea between adults (1/33, 3%) and juveniles (1/9, 11.1%) ($p = 0.54$) controlling for year and location, *Moniezia* between adults (1/33, 3%) and juveniles (0/9, 0%) ($p = 0.99$), *Orthostrongylus* between adults (1/33, 3 %) and juveniles (0/9, 0%) ($p = 0.99$), *Eimeria* between adults (1/33, 3%) and juveniles (0/9, 0%) ($p = 0.99$), or dorsal-spined larvae between adults (1/33, 3%) and juveniles (0/9, 0%) ($p = 0.99$) was observed.

When sex-classes were compared, no significant difference in prevalence of the nematode eggs of the superfamily Trichostrongyloidea between adults (1/25, 4%) and juveniles (1/17, 5.9%) ($p = 0.67$) when controlled for location and year, *Moniezia* between adults (1/25, 4%) and juveniles (0/17, 0%) ($p = 0.99$), *Orthostrongylus* between adults (1/25, 4%) and juveniles (0/17, 0%) ($p = 0.99$), *Eimeria* between adults (0/25, 0%) and juveniles (1/17, 5.9%) ($p = 0.99$), or dorsal-spined larvae between adults (0/25, 0%) and juveniles (1/17, 5.9%) ($p = 0.99$) was observed.

Between years, there was no significant difference in prevalence of the nematode eggs in the superfamily Trichostrongyloidea between 2007 (1/31, 3.2%) and 2008 (1/11, 9.1%) ($p = 0.99$), *Moniezia* between 2007 (1/31, 3.2%) and 2008 (0/11, 0%) ($p = 0.99$), *Orthostrongylus* between 2007 (1/31, 3.2%) and 2008 (0/11, 0%) ($p = 0.99$), *Eimeria* between 2007 (1/31, 3.2%) and 2008 (0/11, 0%) ($p = 0.99$), or dorsal-spined larvae between 2007 (1/31, 3.2%) and 2008

(0/11, 0%) (p = 0.99) was observed. Differences in prevalence of fecal parasitic eggs, oocysts and larvae were observed among the locations (Figure 7, Table 9). Trichostrongyloidea and dorsal-spined larvae were present in Douglas provincial park, *Orthostrongylus* was detected in Matador community pasture, and *Eimeria*, *Moniezia* and *Orthostrongylus* were detected in Swift Current Creek.

Table 8. Overall prevalence and mean intensity of fecal parasites in 42 white-tailed deer in southern Saskatchewan from winters of 2006, 2007 and 2008

Parasite	Prevalence (%)	Mean	
		Intensity	Range
Nematoda			
Trichostrongyloidea	4.8 (2/42)	0.2 (0.4/2)	0 - 0.2
Orthostrongylus	2.4 (1/42)	0.4 (0.4/1)	0 - 0.4
Dorsal-spined larvae	2.4 (1/42)	0.6 (0.6/1)	0 - 0.6
Cestoda			
Moniezia	2.4 (1/42)	90.4(90.4/1)	0 - 90.4
Protozoa			
Eimeria	2.4 (1/42)	1(1/1)	0 -1
Cryptosporidium	0 (0/24)	NA	NA
Giardia	0 (0/24)	NA	NA
Trematoda	0 (0/16)	NA	NA

NA: not applicable

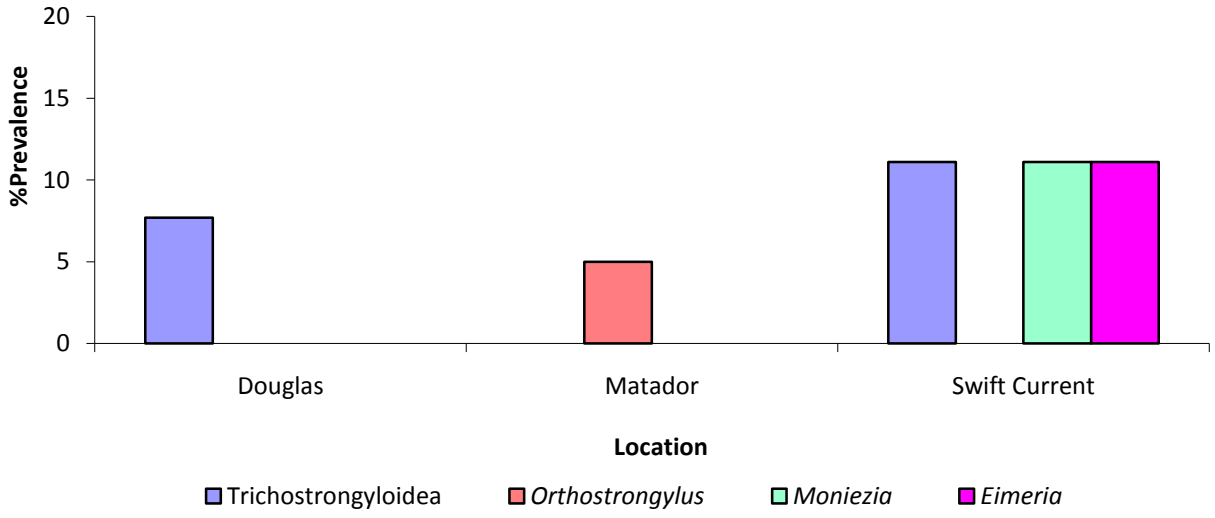

Figure 7. Prevalence of parasites in white-tailed deer in the three study locations in southern Saskatchewan in winters of 2007 and 2008.

Table 9 Prevalence of parasites in white-tailed deer in the three study locations in southern Saskatchewan in winters of 2007 and 2008.

Location	Douglas	Matador	Swift Current
Trichostrongyloidea	1/13 (7.7%)	0/20 (0%)	1/9 (11.1%)
Nematodirus	0/13 (0%)	0/20 (0%)	0/9 (0%)
Orthostrongylus	0/13 (0%)	1/20 (5%)	0/9 (0%)
Skrjabinema	0/13 (0%)	0/20 (0%)	0/9 (0%)
Trichuris	0/13 (0%)	0/20 (0%)	0/9 (0%)
Dorsal-spined larvae	1/13 (7.7%)	0/20 (0%)	0/9 (0%)
Moniezia	0/13 (0%)	0/20 (0%)	1/9 (11.1%)
Thysanosoma	0/13 (0%)	0/20 (0%)	0/9 (0%)
Eimeria	0/13 (0%)	0/20 (0%)	1/9 (11.1%)
Giardia	0/6 (0%)	0/10 (0%)	0/7 (0%)
Cryptosporidium	0/6 (0%)	0/10 (0%)	0/7 (0%)
Trematodes	0/8 (0%)	0/6 (0%)	0/2 (0%)

2.5. Discussion

This study highlights the prevalence of CWD among mule deer of Swift Current Creek and Antelope Creek in southern Saskatchewan. The higher prevalence of CWD detected in Antelope Creek relative to Swift Current Creek suggests the presence of a CWD cluster in the prior location. Studies have reported that prevalence of CWD in free-ranging mule deer and white-tailed deer to be higher in adult males than adult females (Gross and Miller, 2001; Grear et al., 2006). This study agrees with those findings, however, the number of samples tested was insufficient to detect a difference at the level of statistical significance. CWD was not detected in white-tailed deer in any of the study locations. A small sample size of white-tailed deer (n = 42), and the absence of white-tailed deer in the study sample from Antelope Creek may have contributed to this finding.

This study demonstrates the presence of serum Ab against BoHV-1, PI-3, BVDV-1 and *Neospora caninum* in wild mule deer and white-tailed deer populations in southern Saskatchewan. Similar studies in North America have also reported the exposure of wild deer populations to BoHV-1, PI-3 and BVDV-1 (Lamontagne et al., 1989; Sadi et al., 1991; Aguirre et al., 1995). The serological test systems used in this study were designed to detect Ab against pathogens of cattle; hence, the pathogens that result in Ab production may be identical or closely related to pathogens of cattle (Stauber et al., 1980). Prevalence of Ab against an infectious agent indicates exposure to that agent. However, exposure to an infectious agent may not confirm the presence of disease in the reactor or the possibility of transmission of the agent from deer to livestock (Stauber et al., 1980); and vice versa. Similarities in prevalence between both sexes of mule deer and white-tailed deer for the agents tested in this study, as well as differences in prevalence between age classes in mule deer for BoHV-1, PI-3 and BVDV-1, are in accord with

previous studies (Ingebrigtsen et al., 1986; Sadi et al., 1991). Higher prevalence of viral agents in adult deer compared to juveniles is likely related to the longer time period over which exposure and re-exposure could occur and the higher potential for exposure due to greater movement in adulthood (Capman and Early, 2001). The relatively smaller sample size of white-tailed deer in this study may not be sufficient to detect differences between groups of interest.

Ruminants are infected with alphaherpesviruses and gammaherpesviruses (Engels and Ackermann, 1996). Alphaherpesviruses have a variable host range and gammaherpesviruses have a restricted host range (King, 2001). Infectious bovine rhinotracheitis is a respiratory disease of cattle caused by bovine herpesvirus 1 (BoHV-1) of *alphaherpesvirinae* (Dawson et al., 1962). However, several ruminant *alphaherpesvirus*, i.e. caprine herpesvirus 1 in goats, cervid herpesvirus 1 in deer, cervid herpesvirus 2 in reindeer (*Rangifer tarandus*), elk herpesvirus 1 in elk, and bubaline herpesvirus 1 in buffaloe (*Bubalus bubalis*) have been shown to be antigenically related to BoHV-1 (Lyaku et al., 1992; Thiry et al., 2006). Anti-viral Ab of different *alphaherpesvirus* cross-react with BoHV-1 and interfere with SNT and ELISA (Lyaku et al., 1992). Because of this, Ab against BoHV-1 in mule deer and white-tailed deer serum in this study could be more precisely interpreted as an exposure to any member of the ruminant *alphaherpesvirinae*.

Anorexia, depression, excess saliva secretion and respiratory distress were observed in mule deer when experimentally infected with BoHV-1(Chow and Davis, 1964); however, no clinical signs were observed when a BoHV-1 isolate from red deer (*Cervus elaphus*) was experimentally infected to another of the same species (Reid et al., 1986).

Bovine virus diarrhoea mucosal disease, an economically important disease in cattle and sheep, is caused by BVDV-1 or BVDV-2 (Houe, 2003). Persistently infected domestic cattle and

sheep are reservoirs of BVDV and close contact of wild ruminants with livestock could facilitate the transmission of this agent (Campen and Frolish, 2001). Persistently infected status was experimentally established in two white-tailed deer fawns (Passler et al., 2007; Duncan et al., 2008). Persistently infected white-tailed deer doe had been shown as a source of infection (Passler et al., 2010).When inoculated experimentally with a BVDV isolate, mule deer and white-tailed deer fawns were viremic, and shed virus in nasal secretions but did not show the clinical disease (Van Campen et al., 1997). However, mild diarrhoea, laminitis and coronitis were observed in two reindeer calves experimentally infected with Sanger strain of BVDV (Morton et al., 1990). Detection of the BVDV in nasal secretions of deer in an experimental study suggests the possibility of transmission through close contact (Van Campen et al., 1997). Genomic differences have been identified in a BVDV isolate from a wild roe deer (*Capreolus capreolus*) and a strain isolated from livestock (Frolich and Hofmann, 1995). Further, a 60-70% seroprevalence of BVDV-1 observed in wild caribou (*Rangifer tarandus*) distant from livestock supports the possibility of maintaining a virus that cross reacts in wild caribou (Elazhary et al., 1981).

Parainfluenza viruses cause upper respiratory tract infections in a variety of wild and domestic animals (Capman and Early, 2001). Typically infections are sub-clinical and uncomplicated but interstitial pneumonia can occur in wild ruminants (Capman and Early, 2001). Various species of livestock are reservoirs of PI-3; however, PI-3 has been reported in a wild deer population that had no direct or indirect contact with livestock for over 50 years (Sadi et al., 1991). Due to its contagious nature and aerogenous transmission, PI-3 infects over 80% of wild ruminants, and Ab against PI-3 is frequently detected in these populations (Stauber et al., 1980; Sadi et al., 1991; Capman and Early, 2001). In the present study, detection of Ab against

PI-3 consistently, at a relatively high prevalence, suggests frequent exposure of wild deer to PI-3; and re-infection or prevalence of this virus in deer herds as sub-clinical infections (Capman and Early, 2001).

Neospora caninum has been identified as an important cause of bovine abortion (Dubey, 2003). The life cycle of *Neospora caninum* takes advantage of predator-prey relationships between definitive and intermediate hosts. In the domestic life cycle, domestic dogs (*Canis familiaris*) are the definitive host of *Neospora caninum*, and pasture and water sources contaminated with feces containing *Neospora caninum* oocysts act as the source of infection to cattle, the intermediate host. In cattle herds, infections of *Neospora caninum* is maintained by trans-placental transmission (Dubey et al., 1996). Systemic neosporosis was reported in a California black-tailed deer (*Odocoilus hemionus columbianus*) fawn (Woods et al., 1994). *Neospora caninum* was isolated from a naturally infected white-tailed deer in the USA (Vianna et al., 2005). Serum Abs against *Neospora caninum* in white-tailed deer was reported in North America (Dubey et al., 1996; Lindsay et al., 2002). However, in Canadian wildlife, a sylvatic cycle of *Neospora caninum* is maintained between coyotes (*Canis latrans*) and white-tailed deer (Rosypal and Lindsay, 2005). Molecular biological studies on internal transcribed spacer region 1 (*ITS-1*) of *Neospora caninum* revealed that the same strain is responsible for both the dog - cow domestic cycle and coyote - white-tailed deer sylvatic cycle (Gondim et al., 2004; Rosypal and Lindsay, 2005). There is serological evidence that a similar association exists between wolf (*Canis lupus*) and moose in North America (Gondim et al., 2004). In Brazil, Ab against *Neospora caninum* have been reported in red fox (*Vulpes vulpes*) and grey fox (*Urocyon cinerioargenteus*) (Rosypal and Lindsay, 2005).

In the present study, the relatively high seroprevalence of *Neospora caninum* in mule deer and white-tailed deer indicates they are potential intermediate hosts in maintaining the sylvatic cycle in southern Saskatchewan. Findings of this study suggest the need to identify the definitive host (such as dogs or wild canids) in maintenance of the *Neospora caninum* life cycle involving wild mule deer or white-tailed deer for future management strategies of the parasite. In addition, the finding that juveniles were seropositive may support vertical transmission of this parasite in deer, as has been described for cattle.

As anti-*Neospora caninum* Ab cross-reacts with *Toxoplasma gondii* when ELISA plates are coated with the whole antigen, the indirect immuno-fluorescent test (IFAT) is recommended to confirm a positive result of *Neospora caninum* (Dubey et al., 1996). However, the commercial competitive ELISA plates used in this study were coated with an immunodominant surface protein 65kDa, which is specific for *Neospora caninum* but not for *Toxoplasma gondii* or *Sarcocysis* species (Baszler et al., 1996). Therefore the seroprevalence detected in this study could be interpreted as exposure of wild deer to *Neospora caninum.*

In wildlife disease surveillance programs, herd Ab profiles are an economical and convenient approach to monitor infections and their epidemiological patterns (Chomel et al., 1994); however, animals that died of infection, as well as those that are infected and have not yet seroconverted, or those with undetectable levels of Ab, are not detected in these serological studies (Philippa et al., 2008). Further, many serum Ab tests that are available for testing domestic animals, have not yet been validated for use in serological studies of most wildlife species (Philippa et al., 2008). Therefore the detected prevalence should be interpreted as an apparent prevalence rather than a true prevalence.

This study generated information on the prevalence of some parasites of mule deer and white-tailed deer in southern Saskatchewan. In these deer populations, Trichostrongyloidea, *Nematodirus*, *Skrjabinema*, *Trichuris*, *Moniezia*, *Thysanosoma* eggs; *Orthostrongylus* and dorsal-spined larvae; and *Eimeria* and *Giardia* oocysts were detected. Trematodes and *Cryptosporidium* species were not detected. Trematodes were not detected in wild deer populations in Saskatchewan between 1969 and 1985; however, *Fascioloides magna* has been detected in wild elk in focal areas of central Saskatchewan (Wobeser et al., 1985). Dry climatic conditions and absence of suitable habitat for the intermediate host (aquatic snail) could be possible explanations for the absence of fluke eggs in the fecal samples tested (Wobeser et al., 1985).

Protostrongyloid parasites have an indirect life cycle involving a terrestrial gastropod as an intermediate host. The *Protostrongylus*-type larvae detected in deer feces could belong to the genus *Orthostrongylus* (Carreno and Hoberg, 1999), possibly the lung worm *Orthostrongylus marcotis* (Pybus, 1990). Dorsal-spined larvae were detected in a fecal sample from a white-tailed deer. Dorsal-spined larvae are produced by several genera of Protostrongylids: *Elaphostrongylus*, *Parelaphostrongylus*, *Muellerius*, *Umingmakstrongylus* and *Varestrongylus* (Lankester, 2001). Dorsal-spined larvae identified in this study were most likely *Varestrongylus alpenae* based on previous reports from white-tailed deer in Saskatchewan (Gray et al., 1985). *Parelaphostrongylus andersoni* has not been reported in Canadian prairies and *P. tenuis* has not been reported west of approximately the Saskatchewan – Manitoba border (Lankester, 2001). However, *P. odocoilei* has been reported in mule deer from Alberta (Lankester, 2001). Molecular biological studies are needed to definitively identify the genus of the detected dorsal-

spined larvae (Hoberg et al., 2005; Jenkins et al., 2005; Huby-Chilton et al., 2006), in white-tailed deer.

In this study, identification of parasites was based on morphological characteristics of eggs, oocyst or larvae, and most specimens were identifiable to the genus level. However, based on morphology "trichostrongyle eggs" could not be identified to genus level because three superfamilies of nematodes belonging to the order Strongylida, namely Trichostrongyloidea, Strongyloidea and Anchylostomatoidea produce morphologically similar strongyle-type eggs (smooth-surfaced ellipsoidal shelled eggs containing a morula) (Bowman, 2009). Adult parasites belonging to the superfamily Trichostrongyloidea (such as genera *Trichostrongylus, Ostertagia, Haemonchus, Cooperia*); and *Bunostomum and Oesophagostomum* inhabit the abomasums, small intestine and large intestine of ruminants and produce "strongyle-type eggs" (Bowman, 2009) in feces which can only be differentiated with molecular techniques (Chilton, 2004). *Nematodirus* species are an exception in that they produce large, distinctive eggs (Foreyt and Foreyt, 2001).

Serveral strongylate nematodes have been reported both in wild mule deer and white-tailed deer : *Haemonchus contortus*, *Ostertagia ostertagi*, *Teladosagia circumcincta*, *Trichostrongylus axei*, *Cooperia oncophora*, *Nematodirus filicollis* and *N. odocoilei*, *Chabertia ovina* and *Oesophagostomum venulosum* (Hoberg et al., 2001). In addition to the above species of strongylate nematodes, *Haemonchus placei, H. simillis, Mazamastrongylus pursglovei, M. odocolei, Obeliscoides cuniculi, Ostertagia mossi, Spiculopteragia spiculoptera, Trichostrongylus askiveli, T. calcaratus, T. dosteri, T. longispicularis, Cooperia cuticei, C. pectinata, C. punctata, C. spatulata, Eucyathostoma webbi, Oesophagostomum cervi, O. columbianum* have been reported in wild white-tailed deer and *Marshallagia marshalli,*

Ostertagia bisonis, *O. leptospicularis*, *Psudostertagia bullosa*, *Cooperia surnabada*, *Nematodirus abomasalis*, *N. helvatianus*, *N. spathiger*, *Nematodirella antelocaprae*, *N. longissimuspiculata*, *Trichostrongylus colubriformis*, *T. longispicularis*, and *T. vitrinus* have been reported in mule deer (Hoberg et al., 2001).Absence of eggs or oocysts in feces does not rule out the presence of parasites, as some persist in adult stages (Fakae, 1990) or in a state of hypobiosis (Capitini et al., 1990) within ruminants in order to survive unfavourable seasons. The shedding of parasite eggs or larvae is sensitive to the seasons of the year as some parasites do not produce, or produce very small numbers of, eggs or larvae during winter (Bowman, 2009), which was when all samples were collected during this study. In a population, only a small number of individuals carry most of the parasitic burden, a phenomenon called aggregation of parasites (Shaw et al., 1998). Thus, the relatively low overall prevalence of most parasitic genera in this study may be associated with winter sampling and aggregation of parasites.

Parasite distribution patterns in a population are influenced by the characteristics of the host species, including body size, diet, social behaviour, population density, group size, habitat and nutrition (Setchell et al., 2007). Within a group, hosts may vary in their susceptibility to parasitism due to differences in age, sex, genotype, health and immune status (Setchell et al., 2007). With increases in host densities, individuals are more likely to become infected due to increased transmission rates (Setchell et al., 2007). Further, testosterone levels in males and status of the reproductive cycle in females may influence infection by reducing immune-competence during particular seasons (Setchell et al., 2007).

In this study, Trichostrongyloidea, *Skrjabinema*,and *Moniezia* eggs, *Orthostrongylus* larvae and *Eimeria* oocysts were detected in fecal samples of deer collected from all study locations. Parasitological prevalence was observed to be significantly higher in juveniles than

adults for *Nematodirus*, *Moniezia*, *Thysanosoma and Orthostrongylus*, and this could be explained with the delay in immunological responsiveness in juveniles (Colditz et al., 1996). Variation was observed in the prevalence of each parasite among years. This agrees with previous reports on variation of prevalence from year to year due to climatic differences (Waller and Thomas, 1978).

Selecting a parasite as a marker in evaluation of the effectiveness of herd reduction in CWD management was based on the mode of transmission, host specificity, direct life cycle and moderate prevalence. Many gastrointestinal parasites have the potential to infect sympatric ungulates (Preston et al., 1979; Zaffaroni et al., 2000; Ezenwa, 2003). As wild deer in these study locations may be exposed to livestock and other wild ungulates, there is potential for sharing parasites between sympatric species. Members of the superfamily Trichostrongyloidea; and genera *Trichuris* and *Moniezia*, and *Neospora caninum* are generalist parasites that are shared among sympatric hosts (Ezenwa, 2003). Therefore the generalist parasites are less likely to be useful as potential tools in the evaluation of management strategies for controlling CWD. Further, *Moniezia*, *Thysanosoma* and *Orthostrongylus* could not be selected as inferential tools in the evaluation of CWD management strategies due to their indirect life cycles. *Eimeria* and *Skrjabinema* species are relatively host specific, and their transmission across sympatric host species is extremely unlikely (Olsen and Tolman, 1950; Levine and Ivens, 1986). Hence, among the prevalent parasitic fauna in this study, *Eimeria* and *Skrjabinema* could be identified as potential tools in the evaluation of management strategies adopted to control CWD. However, the prevalence of these parasites is reported to be sensitive to season of the year. Eggs of *Skrjabinema* will not necessarily passed in deer feces because the adult pin worm lay eggs on the

perianal skin. Based on these observations *Eimeria* is a better candidate than *Skrjabinema* as a marker.

2.6. Conclusion

The information generated in this study could be used in designing wildlife management strategies in southern Saskatchewan. Continuous interactions among infected and susceptible individuals with deer herds and the exchange of pathogens at the livestock - wildlife interface may have contributed to the prevalence of CWD, viral agents and parasitic agents among deer populations. Herpesviruses, PI-3, BVDV-1, *Eimeria* and *Skrjabinema* are identified as potential tools in the evaluation of current management strategies in controlling CWD. Attempts should be made to isolate these viral agents and identify *Eimeria* and *Skrjabinema* at the species level. Further studies using molecular methodologies should be carried out on *Eimeria, Skrjabinema,* herpesviruses, PI-3 and BVDV-1 with the goal of identifying specific viral agents or parasites of deer as potential tools in the evaluation of management strategies used to control CWD in southern Saskatchewan.

3.0. EVALUATION OF MULE DEER LYMPHOTROPIC HERPESVIRUS AS AN INFERENCIAL TOOL FOR UNDERSTANDING HOST POPULATION STRUCTURE AND DISEASE TRANSMISSION IN MULE DEER (*Odocoileus hemionus*) AND WHITE-TAILED DEER (*Odocoileus virginianus*)

3.1. Abstract

A serological survey of mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) from several study locations in southern Saskatchewan during the winters of 2006, 2007 and 2008 detected exposure to a herpesvirus. Pan-herpes PCR on pooled retropharyngeal lymph nodes and trigeminal ganglia of wild white-tailed and mule deer revealed a prevalence of 42.1% (40/95). DNA sequences of eight, 215 pb pan-herpes amplicons confirmed the detected sequence to be 98 - 100% identical to the partial sequences of DNA polymerase gene of type 2 ruminant rhadinovirus of mule deer or mule deer lymphotropic herpesvirus (mule deer-LHV). Genome walking was performed upstream from the amplified sequence of 215 bp and a 3.6 kb contiguous sequence was generated which included the 5' end of the DNA polymerase gene, an intergenic spacer region (ISR) and the 3' end of the glycoprotein B (gB) gene (GenBank Accession HM014314). Specific primers were designed to amplify the ISR of mule deer-LHV and these were used to test buffy coat samples from 159 mule deer and 24 white-tailed deer. The overall prevalence of mule deer-LHV in mule deer was 42.1%. The prevalence of mule deer-LHV was significantly higher in 2007 (57/119, 47.9%) compared to 2008 (10/40, 25%) (p = 0.008; OR = 2.78, 95% CI = 1.23 -6.25). No significant difference in prevalence of mule deer-LHV was detected between adults (31/82, 37.8%) and juveniles (36/76, 47.4%) (p = 0.15), females (28/77, 36.4%) and males (39/82, 47.6%) (p = 0.10) or among Antelope Creek (16/36, 44.4%), Beechy (6/18, 33.3%), Douglas (19/36, 52.8%),

Matador (11/39, 28.2%) and Swift Current Creek (15/30, 50%) (p = 0.22) when controlling for year. The overall prevalence of mule deer-LHV in white-tailed deer was 33.3%. The sample size was inadequate to draw conclusions regarding differences in prevalence among various age groups, sex classes or locations.

Specific primers were also designed to amplify the partial DNA sequences of the gB gene of mule deer-LHV and used to characterize the sequence differences of mule deer-LHV in retropharyngeal lymph nodes from mule deer and white-tailed deer in wildlife management zones 13, 14, 50 and herd reduction zone 14. Phylogenetic trees were generated from DNA sequences of the ISR and a partial DNA sequence of the gB gene of mule deer-LHV from mule deer and white- tailed deer of different wildlife management zones in Saskatchewan. Both ISR and partial gB sequences were found to be insufficiently variable and did not consistently cluster according to geographic location. However partial gB sequences of mule deer-LHV identified differences between isolates originating from the host species, mule deer and white-tailed deer. Since mule deer-LHV is prevalent in deer populations, persists in the host in a latent state, it is a potential candidate for understanding the interactions between the hosts. However, identification of a more variable region of the mule deer-LHV genome is required if it could be used as an inferential tool for studying host population structure.

3.2. Introduction

Pathogens can be transmitted either directly between hosts, indirectly through the environment, or through intermediate hosts (Grassly and Fraser, 2008). The distribution of a host population varies spatially and temporally, and may also be divided into demographic groups. Transmission of a disease among animals in a group or among animals of different groups

within a population can reveal patterns and types of interactions among hosts (Whiteman and Parker, 2005). Due to more frequent interactions among spatial neighbours and between individuals of the same demographic group, transmission of infection is most likely to occur between these individuals (Paterson and Viney, 2000). Therefore, understanding the nature of interactions between hosts within a population is an important factor in modeling disease transmission and in developing management and control programs for a particular disease.

In Saskatchewan, CWD is managed in wild deer by herd reduction. Understanding the deer population structure and interactions between individuals are valuable in designing an effective management program in controlling CWD. Low genetic variability within and among populations of many vertebrates makes it difficult to use host genotype alone to understand historical and current, genetic and demographic processes (Whiteman and Parker, 2005). Very little genetic differentiation among sub populations of white-tailed deer was detected in a recent study on population genetic structure of 2000 white-tailed deer sampled from Alberta, British Columbia and Saskatchewan (Cullingham et al., 2011). In contrast, rates of spontaneous mutation in parasites and microbial pathogens are much higher, allowing genotype to be used to understand the structure of their populations over time (Drake and Holland, 1999). When a homologous locus is compared between host and parasite, the rate of evolution of parasite DNA is faster than that of their host (Hafner et al., 1994) Thus, population genetics of these parasites offer an avenue for understanding their hosts' evolutionary history and current demographic processes (Whiteman and Parker, 2005). For example, Biek et al., (2006) derived details of current population structure and interactions in a cougar population using a rapidly evolving feline immunodeficiency virus which infected this population. These details were not apparent when only host genetic data was analysed.

Parasites which produce chronic, persistent infections and those with a direct life cycle, or direct mode of transmission are suitable candidates for the evaluation of host population interactions through parasite population genetics (Whiteman and Parker, 2005). Viruses in particular are attractive candidates because the speed of virus evolution can exceed one million times the rate at which mammals evolve (Seo et al., 2002). Fast evolutionary rates of viruses are an effective survival strategy to evade the host's immune response (Holmes, 2004). The distribution pattern of viruses is controlled by the number and density of susceptible hosts, which changes over time and space (Holmes, 2004). Although RNA viruses are particularly attractive candidates since they have very high evolutionary rates, DNA viruses are also potentially useful since they are more species-specific, have a narrow host range, and demonstrate a pattern of co-speciation with the host (Van Blerkom, 2003).

The family *Herpesviridae* are a group of large, linear, double-stranded DNA viruses (McGeoch et al., 2006), composed of more than 100 members infecting a large range of hosts including: mammals, birds, reptiles, fish and invertebrates (Davison et al., 2005). Based on biological and physical properties, cell tropism, and genome organization, herpesviruses are divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae.* The subfamily *Gammaherpesvirinae* is composed of two genera, *Lymphocryptovirus* and *Rhadinovirus* (Davison et al., 2005). Ruminants are hosts to members of *Alphaherpesvirinae* and *Gammaherpesvirinae* (Engels and Ackermann, 1996). Ruminant rhadinoviruses (RuRV) are subgrouped as malignant catarrhal fever (MCF) subgroup (Type 1 RuRV), non-MCF subgroup (Type 2 RuRV or lymphotropic herpesvirus) and bovine herpesvirus 4 subgroup (Type 3 RuRV) (Li et al., 2005)**.** The RuRV establish latency in lymphocytes in their respective hosts (Davison et al., 2005).

Mule deer lymphotropic herpesvirus (mule deer-LHV) is a member of the non-MCF subgroup (Li et al., 2005). Molecular biological studies and phylogenetic analysis have revealed that mule deer-LHV co-evolved with its natural host, the mule deer (*Odocoileus hemionus*) (Li et al., 2005). Detection of the majority of existing herpesviruses, including mule deer-LHV, has been possible with the development of a consensus primer PCR method, the "pan-herpes PCR" (VanDevanter et al., 1996; Ehlers et al., 1999) which amplifies a 160 – 180 bp DNA fragment of the DNA polymerase (DPOL) gene. As the DPOL gene of the *Herpesviridae* is highly conserved (Ehlers et al., 1999) it may not be a suitable target for detecting variability of different viral isolates within a given host species. However, the sequence of the pan-herpes PCR amplicon is currently the only available sequence of mule deer-LHV genome in the GenBank.

Serological evidence of exposure to herpesviruses in wild mule deer and white-tailed deer has been demonstrated in various locations throughout North America (Aguirre et al., 1995; Lamontagne et al., 1989) and most recently in southern Saskatchewan, (Chapter 2). As herpesviruses co-evolved with their host (Davison, 2002), they could be potential tools to infer deer population structure in southern Saskatchewan which could be useful in understanding the spatial spread of CWD. Understanding population structure of wild deer and patterns of disease spread within and among these populations are important in strengthening the management strategies for CWD in southern Saskatchewan.

A study was conducted in wild mule deer and white-tailed deer in southern Saskatchewan with the following objectives:

1) Identify the herpesvirus which infects deer populations in southern Saskatchewan and to develop a specific conventional PCR for its detection and estimation of its prevalence.

2) Further investigate the potential of the identified herpesvirus in studying the population structure of wild deer in southern Saskatchewan.

3.3. Materials and methods

3.3.1. Sample collection

Retropharyngeal lymph nodes and trigeminal ganglia were collected for detection of herpesvirus from 95 deer heads that were submitted by hunters to the Western/Northern Region of CCWHC, Saskatoon, Saskatchewan, Canada for the surveillance of CWD in fall, 2007 (Figure 8, Table 10). One hundred and eighty-three buffy coat samples collected from mule deer and white-tailed deer in an active surveillance study in southern Saskatchewan in winters of 2007 and 2008 were also used for the detection of herpesvirus (Table 11).

Table 10. Number of pooled retropharyngeal lymph nodes and trigeminal ganglia collected from deer head submissions to Northern/Western Region of CCWHC in fall of 2007

Wildlife management zone	Number of samples	
	Mule deer	White-tailed deer
WMZ-02	1	0
WMZ-10	3	0
WMZ-13	12	10
WMZ-25	1	0
WMZ-29	1	0
WMZ-50	1	16
WMZ-62	0	1
HRZ-14	36	11
HRZ-25	2	0
Total	57	38

WMZ = wildlife management zone; HRZ = herd reduction zone

HRZ 14 = WMZ 12, 13 and 14

HRZ 25 is a part of WMZ 25 in figure 8

Table 11. Buffy coat samples collected from mule deer and white-tailed deer in winters of 2007 and 2008

Wildlife management zone	Study area	Mule deer		White-tailed deer	
		2007	2008	2007	2008
12	Antelope Creek	29	7	0	0
13	Beechy	15	3	0	0
	Douglas	29	7	4	5
	Matador	26	13	4	4
	Swift Current Creek	20	10	7	0
TOTAL		119	40	15	9

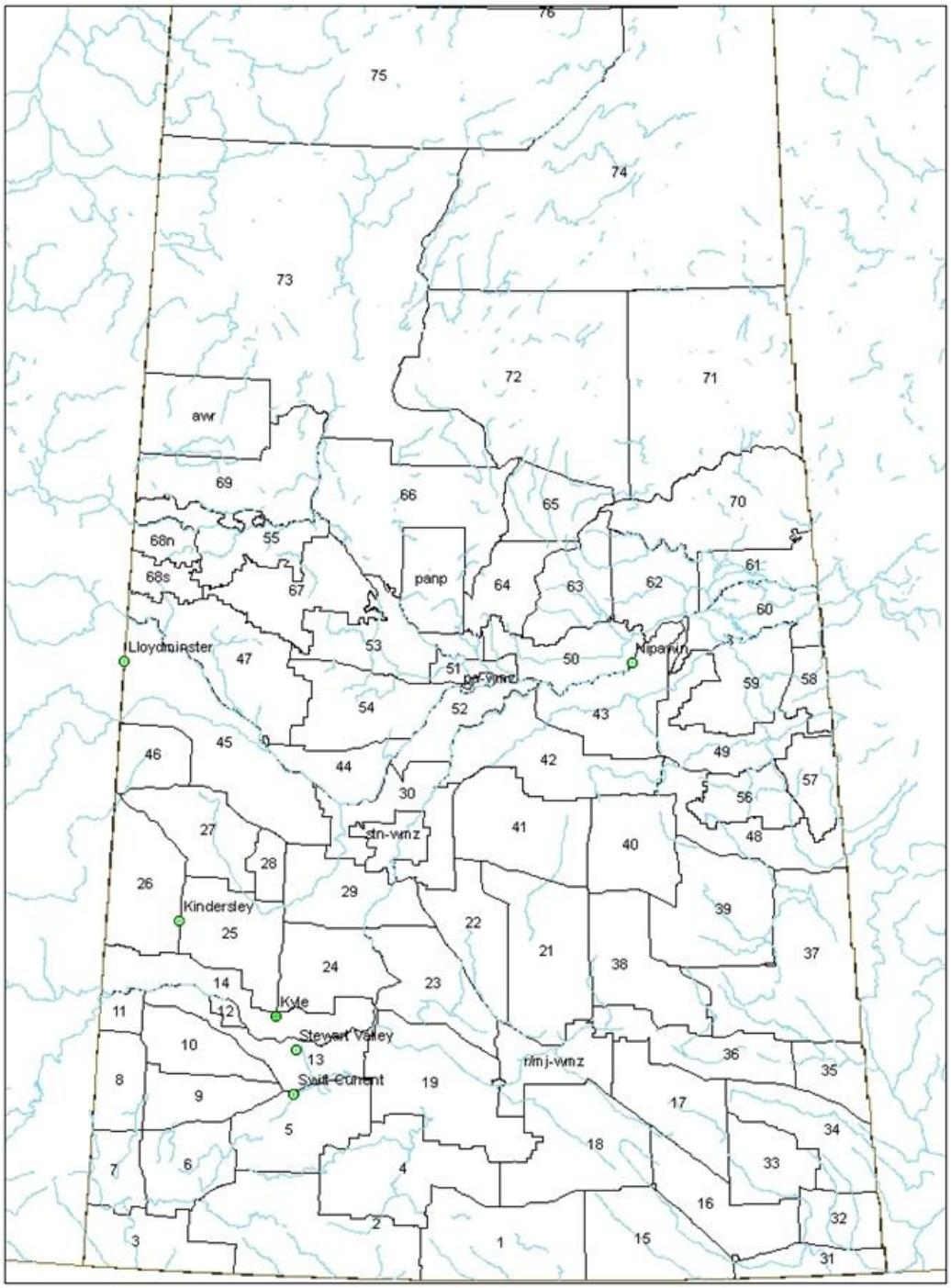

Figure 8. Map of wildlife management zones in Saskatchewan

3.3.2. DNA extraction

A 3×3 mm sized samples from retropharyngeal lymph node and trigeminal ganglion from an individual deer were pooled to detect an alphaherpesvirus or gammaherpesvirus, and DNA was extracted with Qiagen DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA). DNA was also extracted separately to differentiate the herpesvirus, from 3×3 mm trigeminal nerve ganglion and retropharyngeal lymph nodes from 10 deer heads that were positive for mule deer-LHV by the pan-herpes PCR method. The same kit was used to extract DNA from 200 µl of the buffy coat.

3.3.3. Pan-herpes polymerase chain reaction

Amplification of DPOL gene was accomplished by consensus primer nested polymerase chain reaction (PCR) as described previously (VanDevanter et al., 1996). Briefly, 5µl of pooled or individually extracted DNA samples was used as the template in the primary reaction which contained 5 U *Taq* DNA polymerase (Fermentas, Canada), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 250 µM each of dNTPs and 20 pmol each of primers ILK, KGI (two forward primers designed to anneal with 2 conseverd motifs of DPOL gene) and DFA (reverse primer) (Table 12). Reactions were performed in a thermocycler (DNA Engine Peltier Thermal Cycler , MJ Research Inc., MA) using the following conditions: 94 °C for 5 min. followed by 45 cycles of (30 sec. at 94 °C, 60 sec. at 46 °C, 60 sec. at 72 °C) and a final extension of 10 min. at 72 °C. Then, 5 µl of the primary PCR product was used as the template for the secondary PCR in a reaction containing 5 U *Taq* DNA polymerase (Fermentas, Canada) 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 250 µM each of dNTPs and 20 pmol each of primers TGV and IYG (Table 9) under the same cycling conditions. PCR products were visualized on a 1.25% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.4), stained

with ethidium bromide, observed and photographed under ultraviolet light (AlphaImager®, Alpha Innotech, CA). A 100 bp ladder (Invitogen Corporation, Canada) was used as a size standard,

Table 12. Primers

Primer	Sequence (5' – 3')	Description	References
ILK	TCC TGG ACA AGC ARN YSG CNM TNA A	Pan-herpes-1st step	VanDevanter at el.,1996
KGI	GTC TTG CTC ACC AGN TCN ACN CYY TT	Pan-herpes-1st step	VanDevanter at el.,1996
DFA	GAY TTY GCN AGY YTN TAY CC	Pan-herpes-1st step	VanDevanter at el.,1996
TGV	TGT AAC TCG GTG TAY GGN TTY ACN GGN GT	Pan-herpes-2nd step	VanDevanter at el.,1996
IYG	CAC AGA GTC CGT RTC NCC RTA DAT	Pan-herpes-2nd step	VanDevanter at el.,1996
GSP_5	TGT CGC CAT AGA TTA CTT GGA ATC TGG	Genomewalking, gene-specific	This study
GSP_6	AGC ATT GTT CTA CCC CGA AGT GTA ACC	Genomewalking, gene-specific	This study
GSP_7	GTA TAG ACT GGC AAA GTC CAC CAC AAC	Genomewalking, gene-specific	This study
AP1	GTA ATA CGA CTC ACT ATA GGG C	Genomewalking, adapter primer	Genomewalker™ universal kit
AP2	ACT ATA GGG CAC GCG TGG T	Genomewalking, adapter primer	Genomewalker™ universal kit
125F	GAT AGC CTG CGG ACA CAA AT	Sequencing	This study
954R	ACA GGC CAG ATC ACA CAT GA	Sequencing	This study
696F	GTG GCC ACT CTT TTC AGC TC	Sequencing, gB	This study
253R	CCG ATG TCT GTG GTT CAT TG	Sequencing	This study
LHVF	TTA TCG CCC CCT AGA GGA AT	ISR	This study
LHVR	CTA CGT GCC TTG GGC TTA AA	ISR, gB	This study

3.3.4. Genome walking

Polymerase chain reaction is used in genome walking to isolate unknown flanking regions of a known DNA sequence (Rishi et al., 2004). A "genome walking" technique was used to determine the sequence of mule deer-LHV genome upstream of the pan-herpes targeted segment of the DPOL gene. Genome walking was performed with Genomewalker™ universal kit (Clontech laboratories Inc., CA), according to manufacturer's recommendations. Genomic DNA extracted from pooled retropharyngeal lymph node and trigeminal ganglion from a mule deer (ID 70737; positive by pan-herpes PCR and 99% identical to 215 bp of partial DPOL sequence of type 2 RuRV of mule deer or mule deer-LHV), was used as the template for genome walking.

3.3.4.1. Construction of Genome walker libraries

Genomic DNA (2.5 µg) was digested in separate reactions with 80 U, each of *Dra* 1, *Eco*R V, *Pvu* II and *Stu* I (provided with the Genomewalker ™ universal kit) at 37 °C for 18 h. Digested DNA was ethanol precipitated after 1:1 (v/v) phenol chloroform extraction (Sigma-Aldrich Corporation, MO), and DNA pellet was resuspended in 72 µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 7.5). Four microliters of DNA was ligated with Genome walker adapters using DNA ligase (provided with the Genomewalker ™ universal kit), incubated overnight at 16 °C. The constructed DNA libraries were stored at -20 °C.

3.3.4.2. Primers for genome walking

Primers used in the genome walking procedure are given in Table 12. According to Genomewalker ™ universal kit, gene-specific primers GSP_5, GSP_6 and GSP_7 were designed with Primer3 software (Rozen and Skaletsky, 2000) and NetPrimer software (PREMIER Biosoft

International). Gene-specific primers were designed from the sequences close to the end of the known sequences of the partial DPOL gene of mule deer-LHV. The primers were 26 - 30 nucleotides in length, 40 - 60% in G+C content, and had a predicted annealing temperature of 67 °C. Adapter specific primers (AP_1 and AP_2) were supplied with the kit.

3.3.4.3. Nested-polymerase chain reaction in genome walking

Genome walking was achieved with a nested PCR according to manufacture recommendations. PCR reactions were carried out in a thermocycler (PTC-200 DNA Engine Thermal Cycler, MJ Research Inc., MA) separately for each library in a total volume of 50 µl containing 5 µl of 10 × Advantage® 2 PCR buffer (Clontech Laboratories Inc., CA), 1 µl of 50× Advantage® 2 Polymerase Mix (Clontech Laboratories Inc., CA, USA), 1 µl of 50× dNTP mix and 10 pmol of each primers AP_1 and GSP_5 and 1 µl of ligated DNA (genome walker libraries) as the template. PCR conditions for the first round of amplifications were: 7 cycles of 94 °C for 25 sec.; 72 °C for 3 min.; 32 cycles of 94 °C 25 sec.; 67 °C for 3 min. and one additional cycle of 67 °C for 7 min. PCR products from the primary PCR were diluted 1:50 and 1 µl of diluted PCR products were used as the template for the second PCR. The second phase of the nested-PCR were carried in a total volume of 50 µl containing 5 µl of 10× Advantage® 2 PCR buffer (Clontech Laboratories Inc., CA), 1 µl of 50× Advantage® 2 Polymerase Mix (Clontech Laboratories Inc., CA) 1 µl of 50× dNTP mix and 10 pmol of each primers AP_2 and GSP_6.The thermocycler conditions for the secondary PCR were: 5 cycles of 94 °C for 25 sec; 72 °C for 3 min. ; 20 cycles of 94 °C for 25 sec.; 67 °C for 3 min. and one additional cycle of 67 °C for 7 min.

A second round of genome walking was performed following the same protocol with the previously constructed genomic libraries of mule deer (ID 70737), AP_1 and GSP_6 as the first set of primers and AP_2 and GSP_7 as the nested primers (Figure 9). Five microliters of the nested-PCR products were resolved in a 1% (w/v) agarose gel (Sigma-Aldrich, St. Louis, USA) in 1× TE buffer, stained with ethidium bromide along with 1 kb ladder (GenScript Corporation, MA) and observed under ultraviolet light (AlphaImager®, Alpha Innotech, CA). Bands were excised from the gel, purified and eluted with the Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommendations.

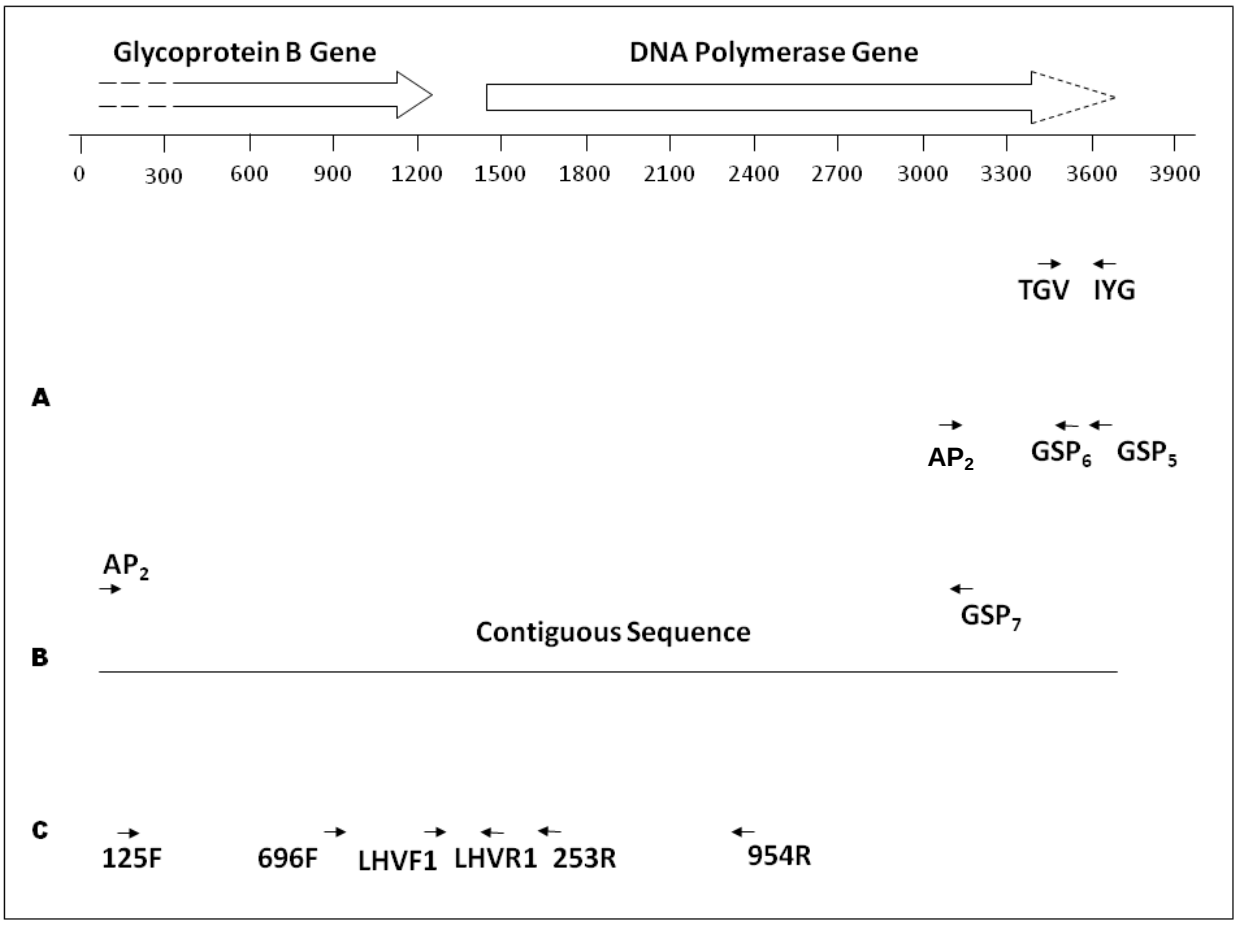

Figure 9. Genome walking map. A = primer landing sites of pan-herpes PCR (TGV, IYG) and genome walking (AP2, GSP5, GSP6 and GSP7); B = contiguous sequence generated from genome walking; C = primer landing sites for sequencing primers (125F, 954R, 696F and 253R) and ISR of mule deer-LHV primers (LHVF1 and LHVR1) and partial gB primers (696F and LHVR1)

3.3.4.4. Cloning of PCR products

PCR products generated from the genome walking were ligated into cloning vector pGEM®-T Easy (Promega Corporation, WI). Prior to ligation, purified PCR product was A-tailed with dATP and *Taq* DNA polymerase to facilitate the base-pairing at ligation to T overhang of the pGEM®-T Easy cloning vector. Briefly, 7 µl of gel-purified PCR product mixed with 1µl of 10× PCR buffer, 1µl of 50mM $MgCl_2$, 0.51µl of 10 mM dATP, and 2.5 U of *Taq* polymerase and incubated at 72 °C for 20 min.

Then, A-tailed PCR products were ligated into cloning vector pGEM®-T Easy. Ligation reactions contained 1µl of pGEM®-T Easy vector, 5µl of 2 × ligation buffer, 3 µl of PCR product, 1 µl of T4 DNA ligase, and the mixture was incubated at 4 °C overnight. Ligation reactions were used to transform HIT™ competent *Escherichia coli* DH5α cells (RBC Bioscience, Taiwan). Transformants were selected on Luria-Bertani (LB) agar plates containing 100 mg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Invitogen Corporation, Canada) and 0.1 mM isopropylthio-β-D-galactoside (IPTG) Invitogen Corporation, Canada). Briefly, LB agar plates were prewarmed to 37 °C, a vial of HIT™ competent *Escherichia coli* DH5α cells were thawed on ice for 10 - 20 sec, and 5 µl of the pGEM®-T Easy Vector ligated PCR product was added, mixed gently by tapping and incubated on ice. Three hundred and fifty microliters of sterile LB broth was added to the mixture, tapped gently and kept in ice. Fifty microlitres, 150 µl and 200 µl of the transformation mixture was spread onto prewarmed individual LB agar plates and incubated at 37 °C overnight. Colonies generated by *Escherichia coli* DH5α cells with the inserted pGEM®-T Easy vector containing PCR product appears as white whereas the *Escherichia coli* DH5α cells containing empty vector appear as blue.

To confirm the presence of inserted DNA in 10 white colonies, a PCR was performed in 25 µl reactions containing 2.5 U *Taq* DNA polymerase (Fermentas Inc., Canada), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 250 µM each of dNTP and 10 pmol each of primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'). Reactions were incubated at 94 °C for 5 min. followed by 40 cycles of (30 sec. at 94 °C, 30 sec. at 55 °C and 30 sec. at 72 °C), and a final extension of 10 min. at 72 °C. Five microliters of the PCR products were visualized under ultraviolet light on a 1% (w/v) agarose gel in 1× TAE stained with ethidium bromide along with 1 kb ladder (GenScript Corporation, MA) as a size standard. PCR confirmed white colonies were picked with a sterile toothpick and inoculated into 5 ml of LB Ampicillin broth and incubated overnight at 37 °C in a shaking incubator at180 rpm. Plasmid DNA was purified from overnight *Escherichia coli* in broth cultures with a spin column kit (EZ-10 spin column, Bio Basic, Canada).

3.3.5. Amplification of 283 bp fragment including the ISR between gB and DPOL genes

A conventional PCR was performed on 2µl of genomic DNA extracted from buffy coat or pooled trigeminal ganglia and retropharyngeal lymph nodes in 50 µl reactions containing 2.5 U *Taq* DNA polymerase (Fermentas Inc., Canada), 2.5 mM $MgCl_2$, 50 mM KCl, 10mM Tris/HCl (pH 8.3), 250 µM each of dNTP and 40 pmol each of primers LHVF1 and LHVR1. Reactions were incubated at 94 °C for 5 min. followed by 40 cycles of (30 sec. at 94 °C, 30 sec. at 60 °C and 30 sec. at 72 °C), and a final extension of 10 min. at 72 °C. Five microliters of the PCR products were visualized under ultraviolet light, on a 1% (w/v) agarose gel in 1× TAE stained with ethidium bromide along with 100 bp DNA ladder as a size standard (GenScript Corporation, MA).

3.3.6. Amplification of a 644 bp fragment including 409 bp gB gene, 177 bp of ISR and 58 bp of the DPOL gene

A conventional PCR was performed on 2µl of DNA extracted from buffy coat or pooled trigeminal ganglia and retropharyngeal lymph nodes as template in a total reaction volume of 50 µl containing 2.5 U *Taq* DNA polymerase (Fermentas Inc., Canada) 2.5 mM $MgCl_2$, 50 mM KCl, 10mM Tris/HCl pH 8.3 , 250 µM each of dNTPs and 40 pmol of each primers 696F and LHVR1. Reactions were incubated at 94 °C for 5 min. followed by 40 cycles of (30 sec. at 94 °C, 30 sec. at 60 °C and 45 sec. at 72 °C) and a final extension of 10 min. at 72 °C . Five microliters of the PCR products were visualized under ultraviolet light on a 1% (w/v) agarose gel in 1× TAE stained with ethidium bromide along with 100 bp DNA ladder as a size standard (GenScript Corporation, MA).

3.3.7. DNA sequencing and analysis

Plasmid DNA or purified PCR products were subjected to cycle sequencing at National Research Council Canada Plant Biotechnology Institute, Saskatoon, Canada. Cycle sequencing was performed using primers T7 and SP6 for plasmid DNA, GSP_6, GSP_7, and AP_2 primer for gel extracted genome walker PCR products. Raw sequence data were processed and assembled with pregap4 and gap4 in the Staden Package (Staden et al., 2001). Edited sequence data were compared with the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database using BLASTn (Altschul et al., 1990). Phylogenetic trees were constructed using the UPGMA method in CLC Sequence Viewer (CLC Bio, Denmark).

3.3.8. Statistical Analysis

The unconditional associations between the mule deer-LHV status based on detection of viral ISR and the potential risk factors, age class, sex and year of observation and location were examined using binary logistic regression (SPSS 16.0 for Windows®, SPSS Inc. Chicago, IL) Risk factors where $p < 0.20$ were identified for consideration in building final multivariable model. All variables that were statistically significant ($p < 0.05$) or potential confounders, defined as variables whose inclusion in the model changed the coefficients of other risk factors of interest by more than 10%, were retained in the model. Where two or more variables were statistically significant, biologically reasonable interaction terms were examined and were retained in the model if the interaction term was statistically significant. Associations were reported as odds ratio (OR) with 95% confidence interval (95% CI).

3.4. Results

3.4.1. Pan-herpes PCR screening of deer heads submitted to Northern/Western region CCWHC, Saskatoon for herpesvirus

Thirty-six percent (21/58) of the pooled retropharyngeal lymph node and trigeminal ganglion samples of mule deer and fifty-one percent (19/37) of white-tailed deer samples yielded a 215 bp amplicon in the pan-herpes PCR assay (Figure 10). DNA sequences of eight pan-herpes positive 215 bp amplicons were 98 - 100% identical to type 2 RuRV of mule deer or mule deer-LHV (GenBank Accession AY 237363). Nine of ten retropharyngeal lymph nodes yielded a 215 bp amplicon of mule deer-LHV, confirming the lymphotropic nature of the virus.

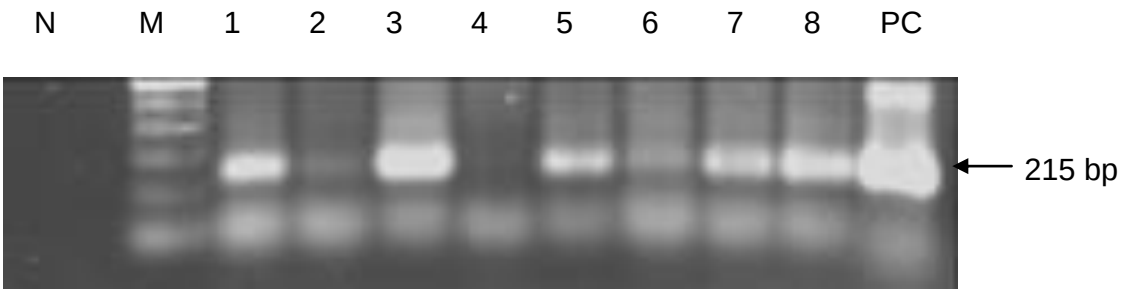

Figure 10. Electrophoresis gel showing PCR products generated with pan-herpes PCR.
Lane N = no template control; lane M = 100 bp molecular marker; lane1- 8 = pooled trigeminal ganglion and retropharyngeal lymph node samples; lane PC = positive control (genomic DNA of infectious bovine rhinotrachitis virus)

3.4.2. Screening for herpesvirus pan-herpes PCR in wildlife management zones of Saskatchewan

Retropharyngeal lymph node samples of mule deer and white-tailed deer from different WMZ and HRZ were selected for surveillance for herpesvirus by pan-herpes PCR. Pan-herpes PCR generated 215 bp amplicon was detected in different management and herd reduction zones: WMZ 10 (2/3, 20%), WMZ 13 (6/22, 27.3%), WMZ 50 (8/17, 47.1%), WMZ 62 (1/1, 100%), HRZ 14 (22/47, 46.8%) and HRZ 25 (1/2, 50%) and not detected in WMZ 2 (0/1, 0%), WMZ 25 (0/1, 0%) and WMZ 29 (0/1, 0%). Ten PCR products were sequenced, and eight of them had quality DNA sequences. DNA sequences were aligned with available sequences in Genbank, and trimmed to 174 bp fragments to match the DNA sequence length of mule deer LHV in Genbank (GenBank Accession AY 237363). These DNA sequences were aligned with multiple sequence alignments in ClustalW (Larkin et al., 2007), and the aligned sequences were visualized in EMBOSS (Rice et al., 2000) (Figure 11). Comparison of 174 bp partial DPOL amplicon sequences derived from three white-tailed and five mule deer of Saskatchewan with those of mule deer-LHV (AY237363) and black-tailed deer-LHV (AY237362) from USA demonstrated few nucleotide substitutions, hence they were insufficiently variable to be used as a tool to detect geographically separated host populations. In these comparisons, a difference in single nucleotide position was revealed between black-tailed-LHV derived from a black-tailed deer in USA and mule deer-LHV of Saskatchewan. The 175 bp partial DPOL sequence of mule deer-LHV from USA differed from that of Saskatchewan in three nucleotide positions. These findings suggest a more variable alternate region of the mule deer-LHV genome would be suitable, if this virus is to be used as an indicator of population structure of the host.

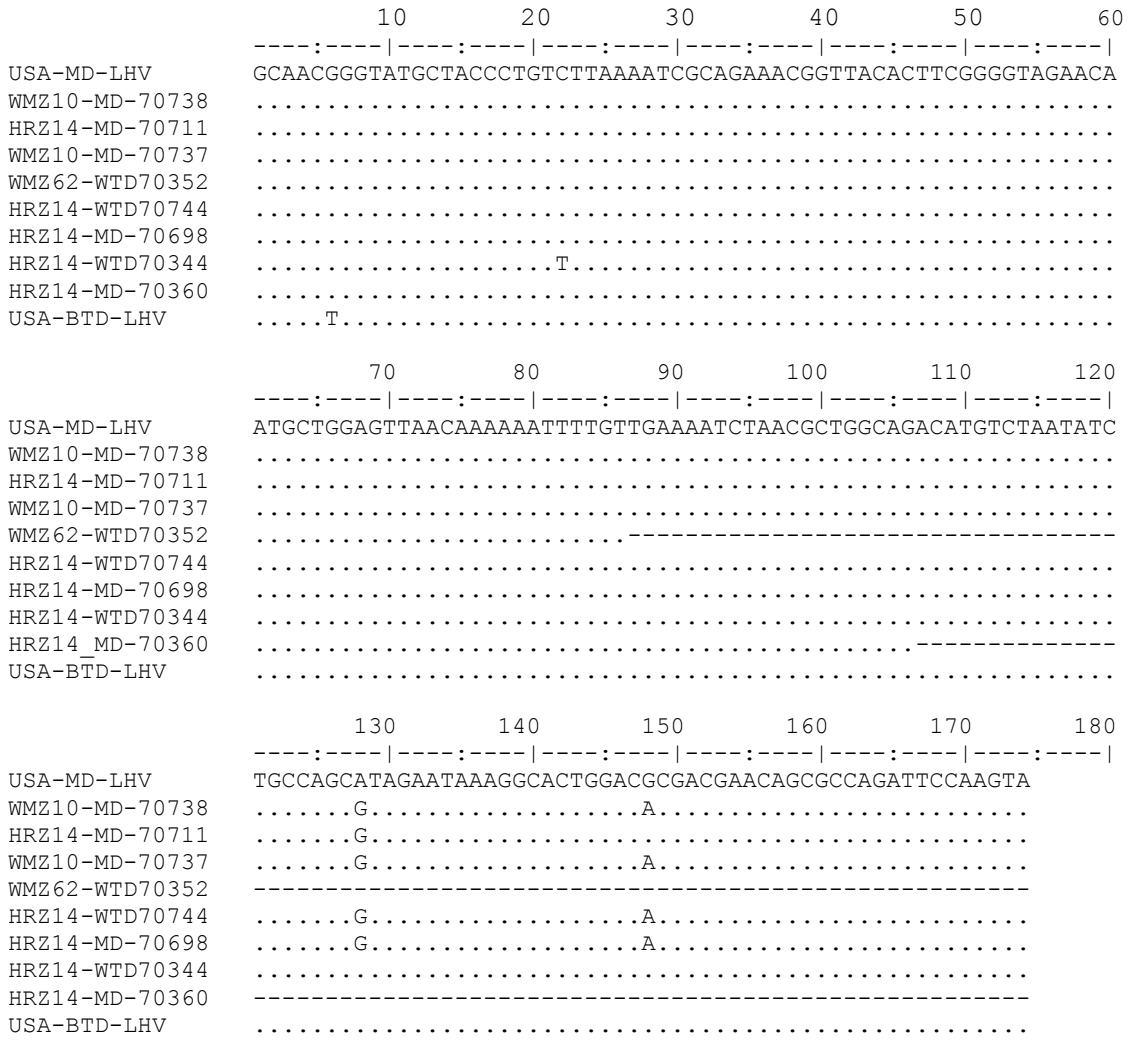

Figure 11. DNA sequence alignment of partial DPOL gene of mule deer-LHV from different wildlife management zones in Saskatchewan aligned with similar sequences from mule deer-LHV (USA-MD-LHV; AY237363) and black-tailed deer-LHV (USA-BTD-LHV;AY237362) from USA. Samples are labelled as: location-species-identification number. Positions of identity are indicated by dots, whereas nucleotide differences are appropriately marked. Partial DNA sequences were indicated as -

3.4.3. Determination of mule deer-LHV genome sequence upstream from the partial DPOL sequence

A genome walking approach was used to determine the genomic sequence of mule deer-LHV upstream from the partial DPOL sequence generated by pan-herpes PCR towards a predicted location of the gB gene, which encodes surface glycoprotein. Analysis of other herpesvirus genome sequences indicated that gB gene is located upstream immediately next to the DPOL gene. It is suggested that the gB gene would have greater genetic variability than the highly conserved DPOL sequence, and therefore, could be used to identify strain differences between species of deer and among locations. Pooled retropharyngeal lymph node and trigeminal ganglion from a mule deer (ID 70737) which was 99% identical to mule deer-LHV was used in constructing genome walker libraries. Amplification of genome walker libraries with gene-specific primer GSP_6 (designed to anneal near the 5' end of the 215 bp partial DPOL sequence produced with primers TGV and IYG) initially resulted an amplicon of a 500 bp DNA fragment from *EcoR* V library (Figure 12, Table 12). A second amplification from a *Pvu* II library using gene-specific primer GSP_7 (designed to anneal near the 5' end of the initial 500 bp product) yielded a 3.4 kb (approximately) fragment (Figure 13,Table 12). The amplified fragments were cloned and sequenced with T7 and SP6 primers, and additional internal sequencing primers (Figure 9, Table 12). A final contiguous sequence of 3690 bp was assembled from the sequence data (Figure 9). Comparison of this 3.6 kb fragment to other published RuRV sequences (OHV-2 and AlHV-1) resulted in the identification of a partial open reading frame homologous to the gB gene, a 177 bp ISR and the partial DPOL open reading frame.

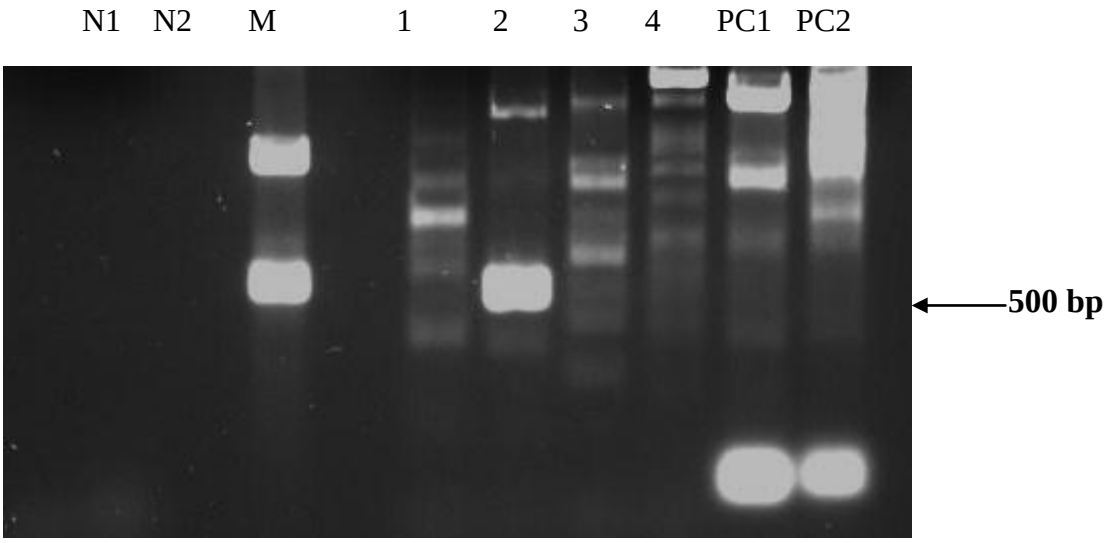

Figure 12. Electrophoresis gel showing PCR products generated with genome walking.
Lane N1= no template control for GSP_6 /AP_2 primers; lane N2 = no template control for the positive controls; lane M = DNA size ladder; lane 1= *Dra* I library; lane 2 = *EcoR* V library; lane 3 = *Pvu* II library; lane 4 = *Stu* I library; lane PC1= genomic library control; lane PC2 = positive control library

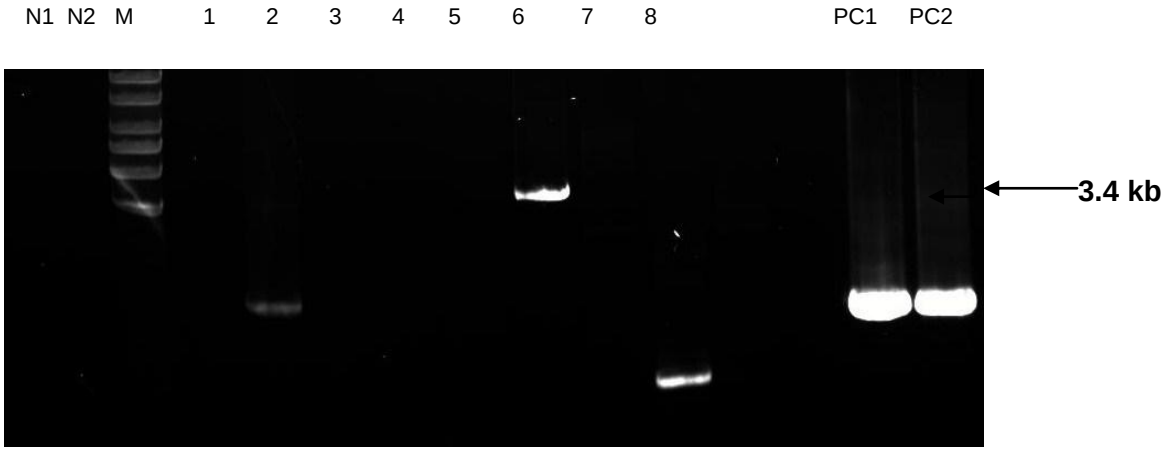

Figure 13. Electropheresis gel showing PCR products generated with genome walking demonstrating a 3.4 kb amplicon of mule deer-LHV genome.
Lane N1= no template control for $GSP_{7,}$ AP_2 primers; lane N2 = no template control to amplify the positive control library; M = DNA size marker; lanes 1and 2 = *Dra* I library; lanes 3 and 4 = *EcoR* V library; lanes 5 and 6 = *Pvu II* library; lanes 7 and 8 = *Stu I* library; lane PC1= genomic library control; lane PC2= positive control library

3.4.4. Design and validation of a specific PCR assay to amplify the ISR of mule deer-LHV

Primers LHVF1 and LHVR1 (Figure 9, Table 12) were designed to amplify a 283 bp DNA fragment, including the entire ISR. The primers amplified the expected product from genomic DNA extracted from pooled retropharyngeal lymph nodes and trigeminal ganglia samples from mule deer-LHV positive deer (70698, 70711, 70737, 70738, 70744) confirming the sensitivity of the assay. Primers failed to amplify the genomic DNA of BoHV-1, BoHV-4 (kindly provided by Prairie Diagnostic Service Inc., Saskatoon) or OHV-2 virus positive tissue from moose (kindly provided by Western/Northern Region, CCWHC) (Figure 14).

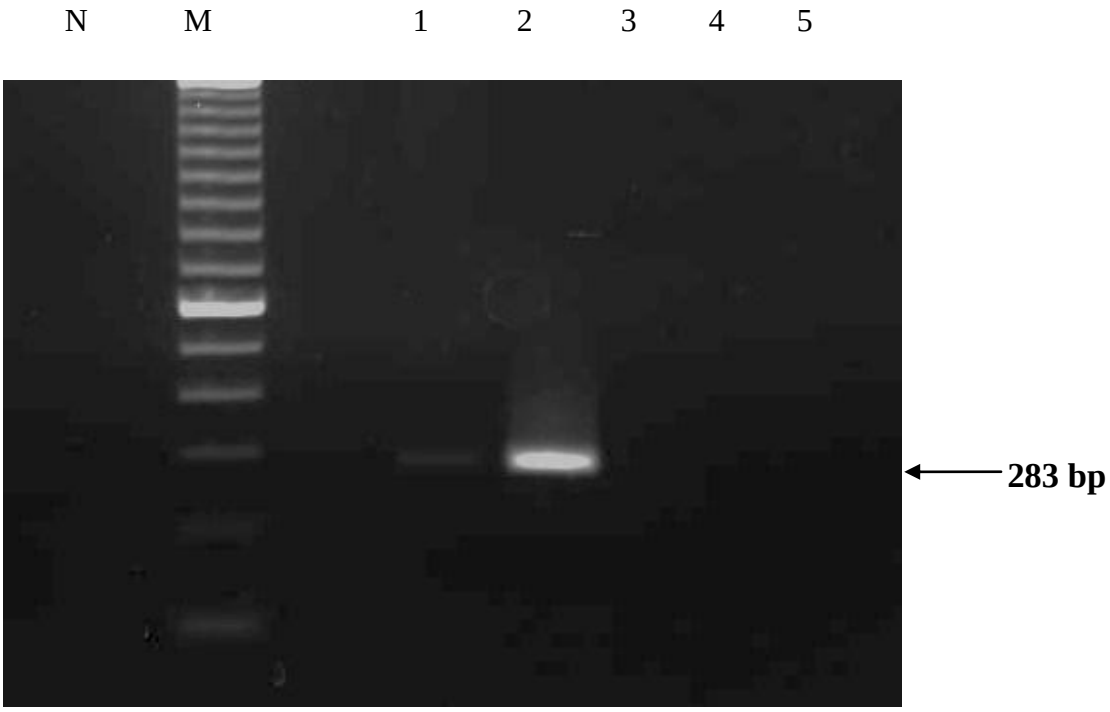

Figure 14. Electrophoresis gel showing specificity of the PCR assay for amplifying ISR of mule deer-LHV. Lane N = no template control; lane M = DNA size ladder; lane 1= genomic DNA of lymph node; lane 2 = positive control plasmid; lane 3 = BHV-1; lane 4 = BHV-4 and lane 5 = OHV-2

3.4.5. Detection of mule deer LHV in buffy coat samples from the active surveillance study

3.4.5.1. Prevalence of mule deer lymphotropic herpesvirus in mule deer

The overall prevalence of mule deer-LHV in mule deer, based on the ISR PCR (Figure 15) was 42.1% (67/159). A significant difference in prevalence of mule deer-LHV between 2007 (57/119, 47.9%) and 2008 (10/40, 25%) ($p = 0.01$) was detected. Samples collected from mule deer in 2007 were more likely to be infected with mule deer-LHV than 2008 (OR = 2.7, 95% CI= 1.2– 6.3). When controlling for year, there was no significant difference in prevalence of mule deer-LHV between adults (31/82, 37.8%) and juveniles (36/76, 47.4%) ($p = 0.29$), females (28/77, 36.4%) and males (39/82, 47.6%) ($p = 0.37$) (a female mule deer in Swift Current Creek was excluded in the analysis due to the unavailability of age record), or among the study locations: Antelope creek (16/36, 44.4%), Beechy (6/18, 33.3%), Douglas (19/36, 52.8%), Matador (11/39, 28.2%) and Swift Current Creek (15/30, 50%), ($p = 0.22$) (Figure 16).

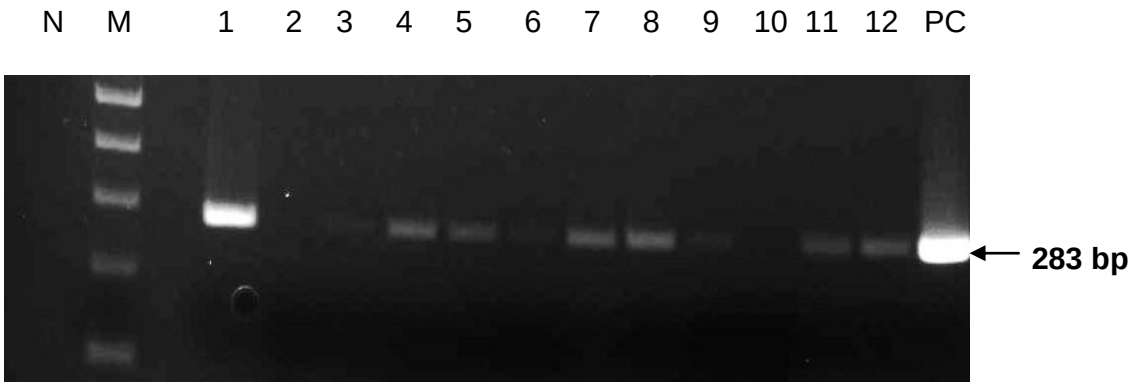

Figure 15. Eectrophoresis gel of conventional PCR for intergenic spacer regions of mule deer-LHV
Lane N = no template control; lane M = DNA size marker; lane 1-12 = genomic DNA extracts from buffy coat samples from wild mule deer and white-tailed deer and lane PC = positive control

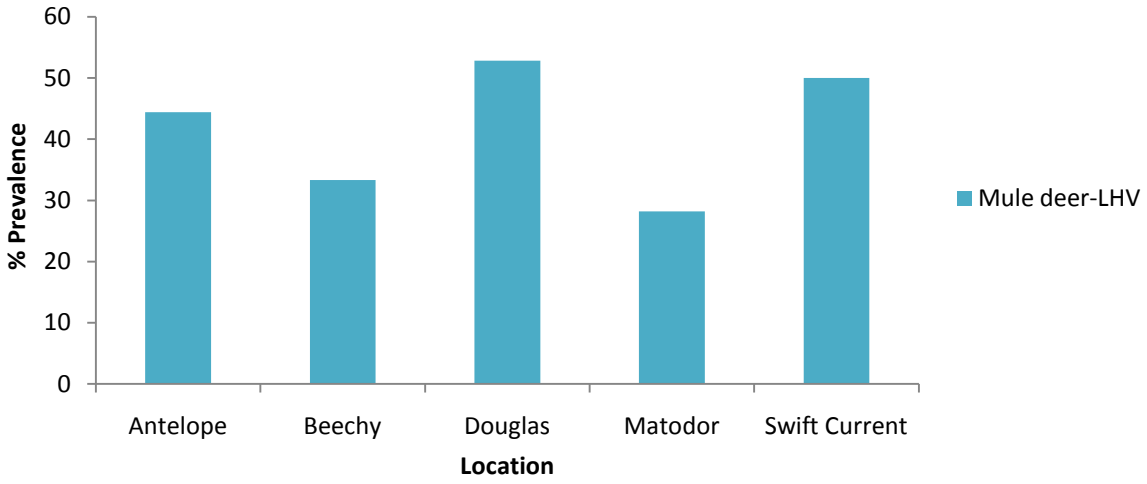

Figure 16. Prevalence of mule deer-LHV in mule deer among study areas in southern Saskatchewan during winters of 2007 and 2008

3.4.5.2. Prevalence of mule deer lymphotropic herpesvirus in white-tailed deer

Overall prevalence of mule deer-LHV in white-tailed deer based on ISR PCR assay was 33.3% (8/24). Prevalence of mule deer-LHV was not significantly different between adults (5/19, 26.3%) and juveniles (3/5, 60%) ($p = 0.18$), females (6/18, 33.3%) and males (2/6, 33.3%) ($p = 0.79$) when controlled for age, 2007 (6/15, 40%) and 2008 (2/9, 22.2%) ($p = 0.34$) when controlled for age, or among study locations: Douglas Provincial Park (1/9, 11.1%), Matador community pasture (4/8, 50%) and Swift Current Creek (3/7, 42.9%) ($p = 0.30$) when controlled for age.

3.4.6. Sequence comparison of ISR of mule deer-LHV derived from lymph nodes and buffy coat samples from mule deer and white-tailed deer from different wildlife management zones

Phylogenetic trees were generated from mule deer-LHV ISR sequences from 28 mule deer and 17 white-tailed deer from 4 different wildlife management zones (WMZ12, WMZ 13, WMZ14 and WMZ 50) and HRZ 14 (Figure 17). This sample consisted of mule deer-LHV sequences from 20 buffy coat and 25 retropharyngeal lymph node samples. The relatively close proximity of HRZ 14 and WMZ 12, WMZ 13, WMZ 14, and the relatively distance of WMZ 50 from other zones (Figure 8) were considered to test the hypothesis that deer populations in geographical areas would be distinguishable by the ISR sequence of mule deer-LHV. Clustering of similar or related sequences was achieved by un-weighted pair-group method using arithmetic averages (UPGMA), and the generated UPGMA tree was used to interpret the results. The phylogenetic analysis of ISR revealed that the sequences did not cluster according to the species of deer or geographic location (Figure 17). The percentage DNA sequence similarity between any given pair of ISR was between 95% and 100%.

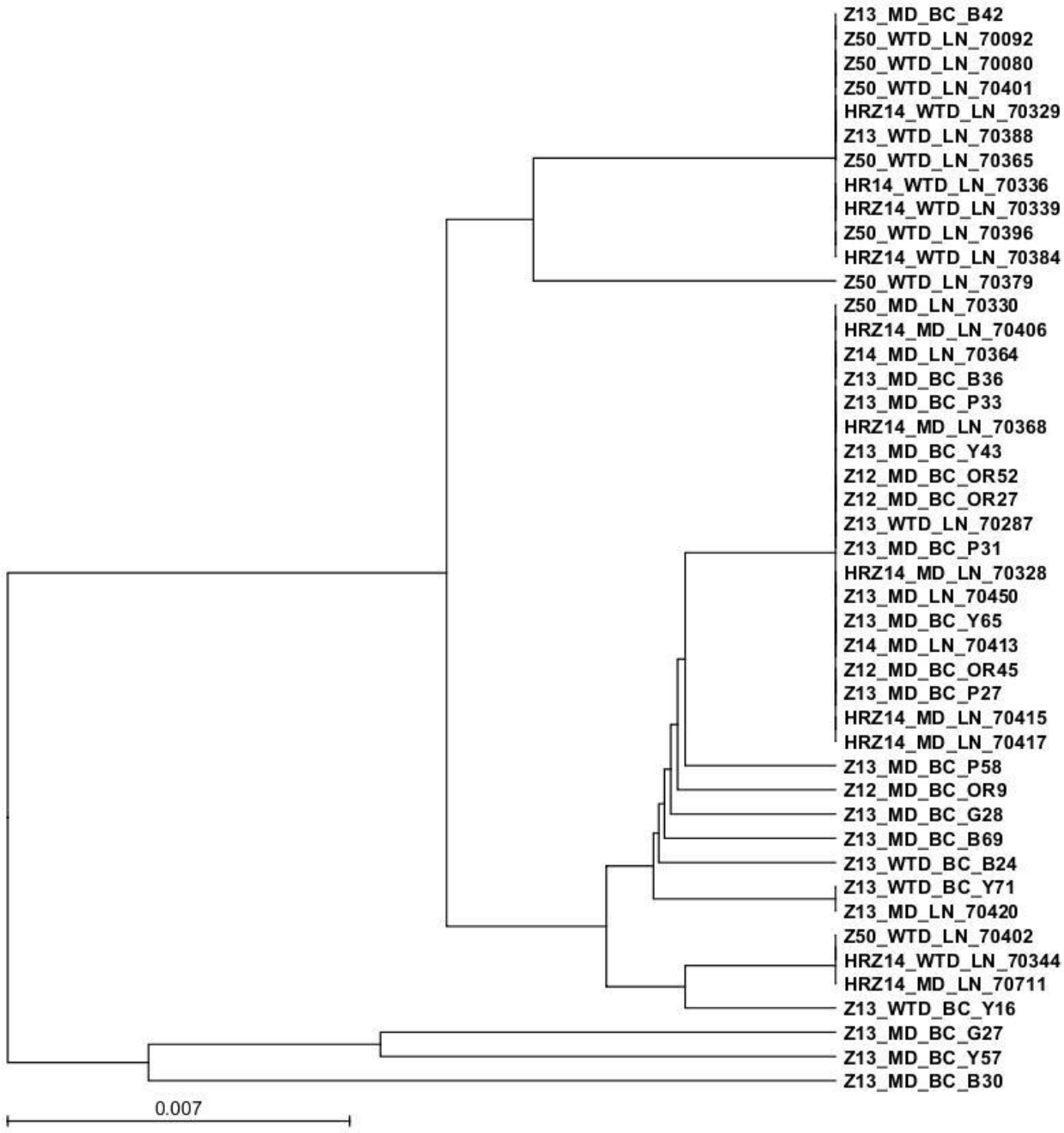

Figure 17. UPGMA tree of intergenic spacer regions of mule deer-LHV from different wildlife management zones. Samples are labelled as wildlife management zone (Z)_species of deer (MD = mule deer, WTD = white-tailed deer)_tissue_(BC = buffy coat, LN = lymph node)_animal identification number

3.4.7. Phylogenetic analysis of partial gB gene and concatenated gB/ISR sequences

Pan-herpes PCR positive retropharyngeal lymph node samples of mule deer (n = 10) and white-tailed deer (n = 18) from wildlife management zones 13, 14 and 50 and HRZ14 (table 13) were amplified with primers 696F and LHVR1 to generate a 644 bp DNA fragment which included the entire ISR (177 bp) and partial sequence of the gB (409 bp) and DPOL (58 bp) gene (Figure 18). The relatively close proximity of HRZ 14 and WMZ 13, WMZ14, and the relatively distant WMZ 50 from other zones (Figure 8) were selected to test the hypothesis that deer populations in geographical areas would be distinguishable by the partial gB sequence of the mule deer-LHV. A 644 bp PCR product was generated from 82% (23/28) of the samples, and 21 of these PCR products yielded high quality sequence data. Clustering of similar or related sequences was achieved by UPGMA, and the generated UPGMA tree was used to interpret the results (Figure 19). Phylogenetic analysis revealed that DNA sequence of the amplified region was insufficiently variable to be used as a tool in identifying geographically separated host populations, and once more, the sequences did not cluster according to geographic origin. However, clustering of sequences based on host species was evident with separation of white-tailed deer and mule deer derived sequences.

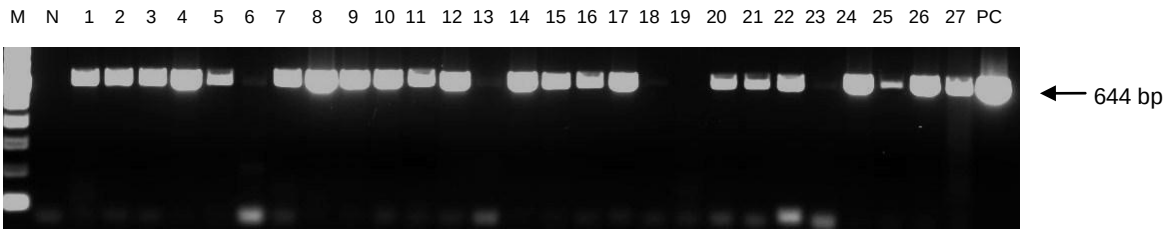

Figure 18. Agarose gel electrophoresis of conventional PCR for the amplification of 644 bp of gB gene from retropharyngeal lymph nodes of deer representing WMZ 13, WMZ14, WMZ 50 and HRZ14.Lane M = DNA molecular weight marker; lane N = no template control; lanes1 - 27 genomic DNA; lane PC = positive control

Table 13.Samples included in phylogenetic analysis of the partial gB gene DNA sequences of mule deer-LHV

WMZ	Mule deer	White-tailed Deer	Total
13	1	3	4
14	0	1	1
50	1	5	6
HRZ 14	7	3	10
Total	9	12	21

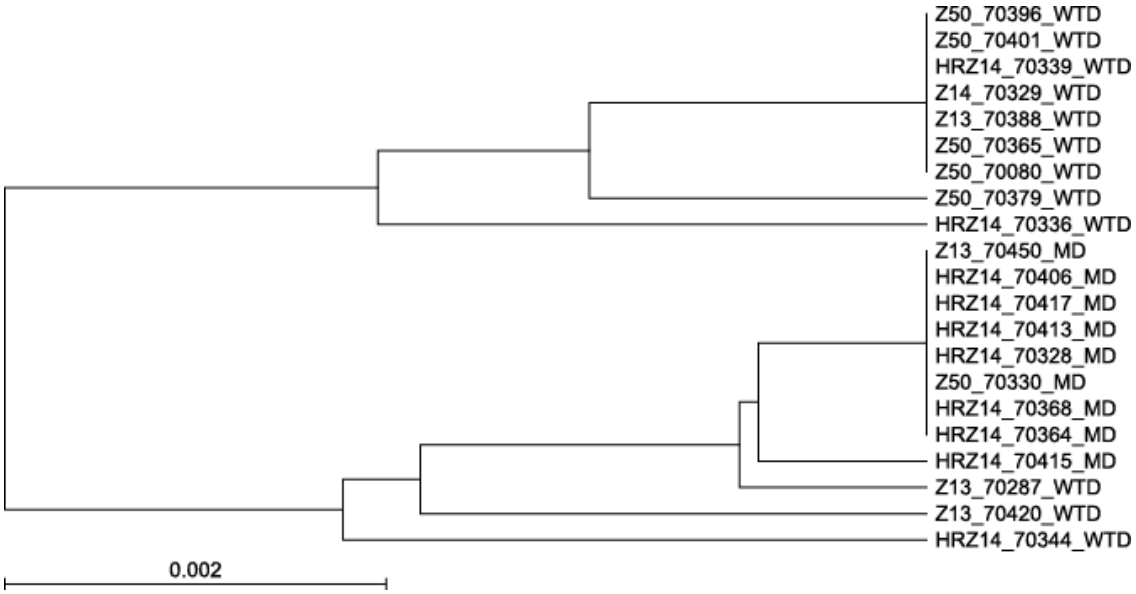

Figure 19. Phylogenic tree of intergenic spacer region and 393 bp of partial gB gene of 21DNA sequences from different wildlife management zones. Samples are labelled as: wildlife management zone (Z)_animal identification number_ species of deer (MD = mule deer, WTD = white-tailed deer)

To determine if the highly conserved ISR sequence was masking informative diversity in the 644 bp gB sequence, the ISR was excluded, thereby resulted a 393 bp fragment of the gB gene for the DNA sequence analysis. Twenty-one 393 bp partial gB sequences of mule deer-LHVgenome were aligned using multiple sequence alignments in ClustalW (Larkin et al., 2007) and visualized in EMBOSS (Rice et al., 2000) (Figure 20). Differences in nucleotide positions were observed in seven locations (Figure 21). A single-nucleotide substitution was observed in three white-tailed deer and one mule deer derived mule deer-LHV sequences. Two nucleotide substitutions were observed in 8/12 of white-tailed deer: from C (cytosine) to T (thymine) at positions 96, and from G (guanine) to A (adenine) at position 102. A single-nucleotide substitution from C to G at position 253 was observed in 9/9 of mule deer derived partial gB sequences.

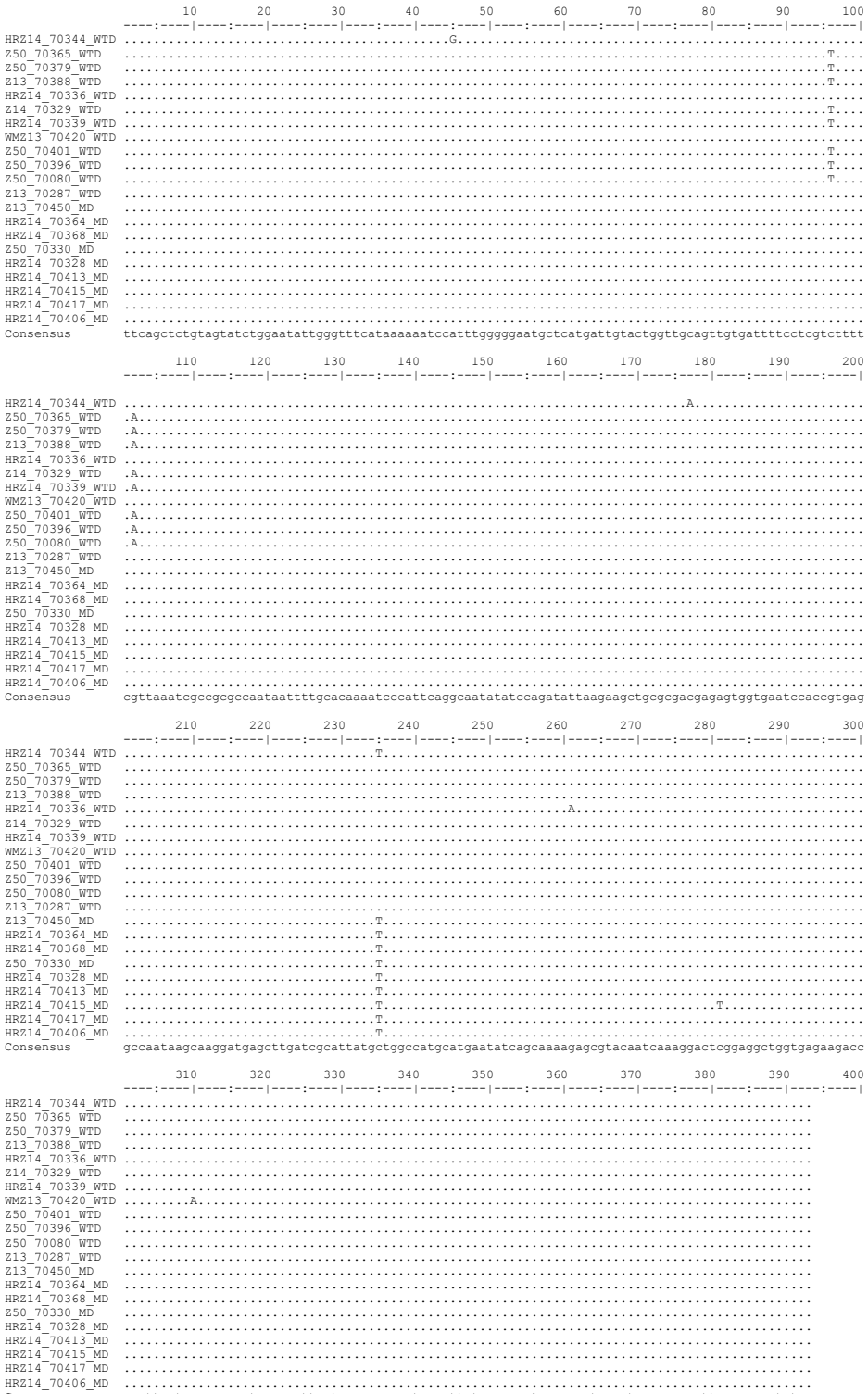

Figure 20. DNA sequence alignments of 393 bp partial gB gene of mule deer-LHV from different wildlife management zones in Saskatchewan. Positions of identity are indicated by dots, whereas nucleotide differences are appropriately marked.

According to the tested hypothesis, partial gB sequences derived from wild deer in WMZ 50 were distinct from the partial gB sequences derived from other WMZ, but such distinction was not observed with the 393 bp of partial gB gene. The partial gB DNA sequence derived from white-tailed deer in HRZ 14 and WMZ 50 were 100% identical. Clustering of sequences of mule deer -LHV based on the host species was observed in the phylogenetic tree for partial gB gene (Figure 21).

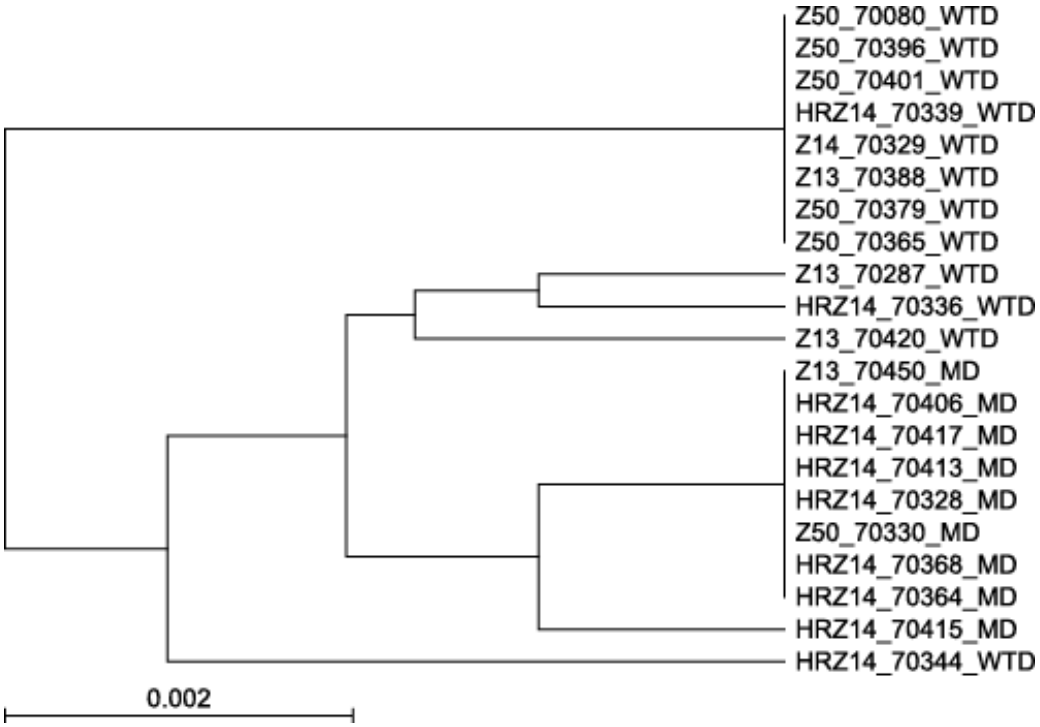

Figure 21. Phylogenic tree of 393 bp of partial gB gene of derived from retropharyngeal lymph node samples from wildlife management zones 13, 14, and HRZ 14
Samples are labelled as: location (Z = zone, HRZ =herd reduction zone) _animal identification number_species of deer (MD = mule deer, WTD = white-tailed deer)

3.5 Discussion

The herpesvirus detected in deer populations in southern Saskatchewan was identified as mule deer lymphotropic herpesvirus (mule deer-LHV); based on the 215 bp sequence of the pan-herpes PCR product (AY237363) (Li et al., 2005). Mule deer-LHV belongs to a subgroup of ruminant rhadinoviruses (Type 2 RuRV) which infects several ruminant species such as cattle, sheep, goats, elk, fallow deer (*Dama dama*) and oryx (*Oryx gazella*). The RuRV are thought to have co-evolved with their hosts and no disease association has been reported (Li et al., 2005).

Although mule deer-LHV has been previously detected from buffy coat of three mule deer and eight black-tailed deer in Washington State, USA (Li et al., 2005), the prevalence in wild populations has not been previously reported. Detection of 42% prevalence by pan- herpes PCR on pooled retropharyngeal lymph nodes and trigeminal ganglia collected in 2007 fall and detection of a similar prevalence by ISR PCR on buffy coat of a different sample of mule deer and 33% prevalence in white tailed deer in 2007 and 2008 winter suggests the maintenance of this agent in southern Saskatchewan deer populations. Detection of mule deer-LHV in buffy coat and retropharyngeal lymph nodes may support the establishment of a latency of the virus in lymphocytes. The same hunter-submitted deer head could be used for both CWD and mule deer-LHV surveillance. Gammaherpesviruses are known to have horizontal transmission, however, the mode of transmission of this virus among deer populations needs to be studied further .The latency facilitates the potential of detecting the agent once infected; therefore this virus could be a potential marker in studying deer population structure and disease transmission among wild deer.

Among the RuRV, the whole genome sequences are available for AlHV-1 (Ensser et al., 1997) and OvHV-2 (Hart et al., 2007). However, information on these aspects in mule deer-LHV

is scarce. Currently, there is no information available on propagation of mule deer-LHV, hence, alternative strategies were attempted in further characterizing mule deer-LHV.

In this study, genome walking method was used to obtain DNA sequence information on mule deer-LHV genome**.** This method involves restriction digestion of genomic DNA, ligation of the fragments to an adapter and uses it as a template for amplifying flanking regions using PCR. However, the success of this method depends upon the presence of a restriction site in a suitable distance from the gene-specific amplification primer. In this study, genome walking generated a 3.6 kb DNA sequence of mule deer-LHV genome extending from pan-herpes DPOL target region to 3' portion of the gB gene including an intergenic spacer region.

DNA polymerase gene is highly conserved among members of the family *Herpesviridae* (Ehlers et al., 1999). Comparison of the eight pan-herpes PCR DNA sequences with NCBI revealed 98-100% identity to mule deer-LHV. Alignment of these partial DPOL sequences with mule deer-LHV (GenBank accession number AY23763) and black-tailed deer-LHV (GenBank accession number AY237362) sequences derived from Washington State, USA, demonstrated very little sequence diversity. Therefore, this 215 bp region of the DPOL gene is not an optimal target for detection of sequence differences of mule deer-LHV in spatially separated deer populations.

Surface glycoproteins of herpesviruses play an important role in the interactions between the virus and host cell including attachment and entry of the virion, fusion, and cell to cell spread (Schwyzer and Ackermann, 1996).They are exposed to selective pressure of the host, and are therefore important targets for the host's immune response (Thiry et al., 2006). Glycoprotein B is the most highly conserved gene of *Herpesviridae* (Pereira, 1994). Because of the large size and highly conserved nature, gB gene is most frequently used to examine genetic distances

among herpesviruses (Ros and Belák, 2002). It is also used to estimate phylogenetic relationships and evolutionary time scales of mammalian herpesviruses (McGeoch et al., 1995). Although DNA sequences of gB gene are highly conserved, sequence variation in the gB gene has been reported in human herpesvirus 7 (Franti et al., 1998; Chan et al., 2003), elephant endotheliotropic herpesvirus (Fickel et al., 2003), and equine herpesvirus 2 (Borchers et al., 1997). Therefore, gB gene was considered as a target for studying sequence variation in mule deer-LHV genome in this study. Further, gB gene is located upstream next to DPOL gene in OVH-2 and AlHV-1.

In the ribosomal RNA (rRNA) operon, the internal transcribed spacer region (ITS) located between the 16S rRNA and 23S rRNA genes has been used to study phylogeny, evolution and molecular diversity of prokaryote and eukaryote microorganisms (Boyer et al., 2001). Sequence length and polymorphisms in the ITS region has been used as tools for identification of bacterial species or strains (Boyer et al., 2001). The non-coding regions located between genes of a viral genome are not exposed to selection pressure (Martin et al., 2006). Therefore, the non-coding ISR between gB and DPOL genes of mule deer-LHV genome was selected as the target in designing specific primers to detect mule deer-LHV. The ISR between gB and DPOL genes of mule deer-LHV, OHV-2 and AlHV-1 were observed to be different from one another in size and DNA sequence. The ISR of mule deer-LHV, OHV-2 and AlHV-1 were 177 bp, 196 bp and 114 bp, respectively. The percentage similarity of the ISR between DPOL and gB gene was: mule deer-LHV to OHV-2 (AY839756), 40%; mule deer-LHV to AlHV-1 (AF005370), 42%; OHV-2 with AlHV-1, 46%. When compared to OHV-2 and AlHV-1, the differences in fragment length and sequence of mule deer-LHV ISR suggest this region as a potential target for detecting the virus.

The conventional PCR developed in this study targeted the ISR between the gB and DPOL genes, and was capable of detecting mule deer-LHV. In contrast to the pan-herpes PCR described earlier, this one-step PCR is economical and uncomplicated. However, validation of this assay was incomplete because of the unavailability of known positive and negative controls.

Phylogenetic analysis allows mapping the genetic relatedness of sequences which contribute in constructing the phylogenetic tree (Vandamme, 2003). The UPGMA trees of ISR and the partial gB gene revealed the incapability of these targets in detecting the DNA sequence differences among mule deer-LHV derived from WMZ 50 and other wildlife management zones.

Most of the DNA sequences of the partial gB gene cluster according to host species, mule deer or white-tailed deer, and was not observed with ISR sequences in UPGMA tree. The partial gB sequence was capable of resolving the host species and this suggests the host specificity of mule deer-LHV. Twenty-one DNA sequences of partial gB gene included in this analysis were not sufficient to draw conclusions.

According to the observations, ISR and partial DNA sequence of the gB gene of mule deer-LHV are insufficiently variable to be used as tool in detecting geographically separated host populations (host population genetic structure), and therefore the designed primers are superior in detection of mule deer-LHV. This highlights the necessity to identify highly variable regions if mule deer-LHV genome is to be used as an inferential tool in studying deer population structure.

Propagation of mule deer-LHV *in vitro* could be attempted by co-cultivation methods (Borchers et al., 1997; Davison et al., 2005). Among the *Gammaherpesviruses*, AlHV-1(Plowright et al., 1960) , equine herpesvirus 2 (Kemeny and Pearson, 1970; Borchers et al.,

1997), and badgerhepesvirus (Banks et al., 2002) have been propagated *in vitro*. Upon successful propagation, sequencing the entire genome of mule deer-LHV, and comparison with known genomes such as AlHV-1 and OvHV-2 could aid in identifying highly variable regions of the virus. Genome walking upstream or downstream from known sequences is an alternative method to obtain more sequence information for this virus. This systematic molecular approach will enable identification of a highly variable region of mule deer-LHV that can used as a tool in understanding deer population structure and disease transmission patterns.

3.6. Conclusion

Mule deer-LHV has several attributes which make it a useful marker to study disease transmission in deer populations in southern Saskatchewan. These include possible horizontal transmission, host specificity, establishment of latency, relatively moderate prevalence, availability of a detection method, and easiness in collection of samples. Identification of a highly variable region within the genome of mule deer-LHV is required in order to be used as an inferential tool in studying deer population structure.

4.0. GENERAL DISCUSSION

Chronic wasting disease has been identified as an important disease in Canadian wildlife because of its potential to reduce cervid populations, and thereby alter ecosystems. It is also important because of the potential to affect human and livestock health, and associated economic ramifications. CWD was first reported in Saskatchewan wild deer populations in 2000 (Bollinger et al., 2008), and in 2004, a national CWD strategy which provided a frame work for developing management policies to reduce the negative impact of CWD was developed by Canadian authorities (Bollinger et al., 2004).

In Saskatchewan, CWD has been managed in free-ranging wild deer by lowering the possibilities of disease transmission through population reduction. The effectiveness of this strategy is evaluated by annual CWD surveillance of hunter-submitted deer heads, a program undertaken by Saskatchewan Ministry of Environment and Western/Northern region of CCWHC (Bollinger et al., 2008). Similar to CWD surveillance in other jurisdictions, evaluating the effectiveness of current management strategies of CWD in Saskatchewan is challenging because of the long incubation period and relatively low overall prevalence of the disease (Williams et al., 2002a). Other than measuring changes in CWD prevalence over time, methods for evaluation of CWD management effectiveness are required. Evaluating changes in prevalence of other infectious agents that are transmitted in a manner similar to CWD, but those respond more rapidly to management actions may be useful in predicting longer term effects of these actions on CWD.

To find alternatives to the evaluation of CWD management strategies, a study of southern Saskatchewan deer populations was undertaken during the winters of 2006, 2007 and 2008 with the following objectives:

1) determine the prevalence of CWD and other infectious agents in wild deer

2) identify infectious agents that may respond rapidly to herd reduction

3) identify infectious agents that could be potentially used to study pathogen spread and deer population structure

This study revealed that wild deer populations in southern Saskatchewan were found to be infected with, or previously exposed to, CWD and a number of viruses and parasites. Among study locations, CWD was detected in Antelope Creek and Swift Current Creek, with a higher prevalence in the prior location. Antelope Creek also showed the highest prevalence for PI-3. Parainfluenza 3 could be transmitted among deer as a result of transmission within populations (Sadi et al., 1991) or spill-over from sympatric cattle (Capman and Early, 2001). The higher seroprevalence in Antelope Creek may be associated with more frequent and intimate contact between deer in this area relative to other areas, and if true, also may partially explain the higher prevalence of CWD in this location. A similar relationship was not found for parasitic infestations in Antelope Creek which would reflect environmental transmission of infectious agents. Parainfluenza-3 holds promise as an agent for monitoring response to disease management programs.

Further, this study identified the seroprevalence to a herpesvirus and molecular biological studies revealed widespread occurrence of a gammaherpesvirus, mule-deer LHV, in southern Saskatchewan deer populations. A conventional PCR assay was developed to detect the ISR of

mule deer-LHV. This PCR assay was able to detect mule deer-LHV from buffy coat of live deer, and retropharyngeal lymph nodes of hunter-submitted deer heads.

This study generated a partial genomic DNA sequence of mule deer-LHV, including a 2.2 kb segment of DPOL gene, a 1.2 kb segment of gB gene, and a 177 bp segment of ISR between gB and DPOL genes. In phylogenetic analysis of mule deer-LHV, ISR failed to demonstrate clustering of DNA sequences associated with species of deer or geographic location. Because of the low variability of DNA sequences, lower percentage identity to the same target in AlHV-1 and OVHV-2, and confirmed specificity to BoHV-1, BoHV-4 and OHV-2, the ISR of mule deer-LHV is a suitable target for detecting mule deer-LHV in deer populations. Phylogenetic analysis of the mule deer-LHV partial sequence of gB gene revealed sequences clustered into mule deer and white-tailed deer genotypes, however, this target did not detect differences in genotype among geographically distant wild deer populations.

Minimal DNA sequence diversity was observed both in ISR and partial gB gene of mule deer-LHV. Hence, it is recommended that a highly variable region of this virus would be more appropriate to be used as a tool in detection of sequence differences among spatially separated deer populations. This could be achieved by co-cultivation of mule deer-LHV followed by whole genome sequencing or genome walking to obtain sequences of highly variable regions which are expected to be located in the periphery of the genome.

Based on possible horizontal routes of transmission, relative host-specificity, ease of sample collection, and prevalence across study locations; parainfluenza 3 virus, mule deer-LHV, *Eimeria,* and *Skrjabinema* appear to be agents suitable for evaluating the effects of disease management programs in wild deer. Prevalence of these infectious agents should change rapidly if management programs resulted in reduced transmission. This information could be used to

develop and evaluate CWD management strategies; however, due to inherent characteristics of CWD, a decline in prevalence may not be evident for several years after programs are initiated. Because of lower overall prevalence of *Eimeria* and *Skrjabinema* across study locations compared to mule deer-LHV, a larger sample size is required for these two infectious agents to detect changes over time.

In conclusion, parainfluenza 3, mule deer-LHV, *Eimeria* and *Skrjabinema* were identified as potential candidates for evaluation of the effectiveness of CWD management programs in wild deer populations in southern Saskatchewan. Identification of a highly variable region in the genome of mule deer-LHV is required to use it as an inferential tool to study deer population structure and make predictions on spatial spread of CWD.

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